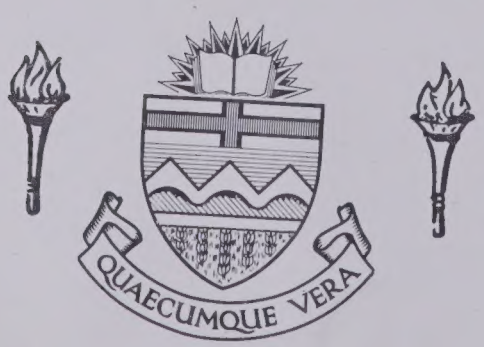

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STERILITY MUTANTS IN *NEUROSPORA CRASSA*

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled"Sterility mutants in *Neurospora crassa*" submitted by Norman V. Vigfusson, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Date .....

ABSTRACT

By means of ultraviolet irradiation of *Neurospora crassa* macroconidia and appropriate selection procedures, 32 mutant strains exhibiting male sterility have been isolated. Both mating types were irradiated and, based on the total number of isolates tested, the *A* mating type showed a 5-fold greater frequency of mutation to male sterility than the *a* mating type. The mutants exhibited a wide spectrum of sexual behavior patterns when used as the male parent in crosses with a wild type strain indicating that the sexual development cycle is blocked at various stages in the different strains. Attempts to restore fertility by increasing the concentration of spermatia in crosses, by varying the incubation time and temperature or by adding various substances to the crossing medium failed.

On the basis of complementation and intergenic recombination data and limited cytological observations, 4 genes are reported which block 'early' stages of sexual development (i.e., prior to karyogamy). Similarly, at least 3 genes are indicated which show blocks at later stages of development. In addition, a gene controlling female fertility was found in 5 out of 32 strains tested, the mutant gene resulting in complete female sterility. This gene segregates independently from male sterile genes which block early stages of the sexual cycle. This independent control of male and female sterility in the early stages of sexual development can be anticipated.

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INTRODUCTION

"The diversity of sexual processes among the fungi has been, for the better part of a century, one of the more intriguing and more intensively investigated aspects of this group of plants. Hypotheses seeking to explain the control of sexual initiation, progression, and consummation, . . . has progressed from the generalized concept of 'ferments', 'humors', and 'potencies' to the demonstrated activites of enzymes, sexual hormones, and genes. Much, however, remains to be done, ..." (Raper, 1960, p. 794). Although life cycles and morphological differentiation of 'sex' in some fungal species were known by the turn of the century, the necessary impetus to the study of sexuality in fungi was not provided until 1904 when Blakeslee demonstrated that the union of two thalli of opposite sexual types was necessary for zygospore formation in *Rhizopus nigricans*. The two 'sexes' in this species were indistinguishable morphologically and were designated (+) and (-). The requirement for sexual interaction of two self-sterile individuals (obligatory cross mating) was termed heterothallism (Blakeslee, 1904) while its counterpart, homothallism, referred to situations where each individual was completely self-fertile. A few years later, Burgeff (1912) demonstrated that a pair of alleles was responsible for the (+) and (-) sexual types. The obvious conclusion at that time was that, in the fungi, the difference between the two was entirely sexual as in any other sexually reproducing organism known at that time. As Blakeslee (*loc. cit.*) had originally stated, the situation was comparable to dioecism and monoecism in higher plants.

This position was soon challenged by the discovery of self-sterile strains in *Glomerella* (Edgerton, 1912, 1914) which were also cross-fertile. Subsequently Kniep (1920) reported a complex sexual pattern in *Schizophyllum commune* which he interpreted as resulting from the independent segregation of alleles at two loci, a condition now referred to as tetrapolar sexuality. In the next decade sexual reproduction was described in many other groups of fungi by a number of workers. This work has been summarized in some excellent review articles notable among which are those by Kniep (1928), Gäumann and Dodge (1928), and Link (1929). The product of all these endeavors was the realization that sexual patterns in fungi were extremely diverse. Heterothallism was described in many fungi representative of all groups and homothallism was found to be equally well distributed. Genetic controls over cross-mating were found to be varied and ranging from sexual dimorphism to complex incompatibility systems. In addition, according to our present knowledge, fungal life cycles vary genetically with regard to the actual duration of their gametophytic and sporophytic generations. Superimposed over this complexity, is a second genetic control responsible for normal sexual development.

Especially in the early years, a clearer understanding of sexuality in fungi has been hampered due to confusion over terminology. Firstly, it became obvious that a strictly dichotomous division of the fungi into homothallic and heterothallic categories was not possible because many species could not be accommodated within this system. Consequently, novel terms have been added over the years

such as secondary homothallism (Dodge, 1927), morphological and physiological heterothallism (Whitehouse, 1949), and mono- and bithallism (Ahmad, 1954) to name but a few. The resulting confusion in terminology is still present and some workers such as Burnett (1956) and Esser (see Raper and Esser, 1964, pp. 216-221) have suggested a complete revision of terms as the only solution.

Secondly, the term 'sexes' has led to confusion. Blakeslee (1906) believed that the (+) strain represented the female and the (-) strain the male. This association, however, was subsequently shown to be inconsistent (Satina and Blakeslee, 1929). Later Gwynne-Vaughan and Williamson (1932) and Drayton (1932) clearly demonstrated in *Ascobolus magnificus* and *Sclerotinia gladioli* respectively, that the difference between the two mating strains was not one of sex as both strains produced male and female sexual organs. The term mating type was soon adopted to designate two obligatory cross-mating individuals. This term does not bear any implications to sex. Sexual factors determine the formation of differentiated male and female sexual organs and/or gametes. Incompatibility factors, on the other hand, determine the mating potential of the individual. In the heterothallic fungi these two factors are utilized (singly or in combination) to promote genetic recombination through the prevention of self-fertilization. Raper (1960) refers to this as the primary genetic control over sexual reproduction in the fungi.

The fungi have evolved two mechanisms by which to exert this control. One of these is sexual dimorphism: the occurrence

of two distinct types which may be designated male and female. Such sexual differentiation is assumed to be due to the segregation of sexual factors although there is as yet no experimental evidence to substantiate this assumption. Sexual dimorphism in the fungi is relatively rare being almost entirely confined to the aquatic Phycomycetes and a few Ascomycetes such as *Ascosphaera apis* (Spiltoir, 1955) and *Ceratocystis fimbriata*(Webster, 1967).

The more common method of prevention of self-fertilization in the fungi is by means of an incompatibility system. As previously stated, this determines the sexual capacities of the individual by segregation at meiosis of certain incompatibility factors. This in turn leads to differentiation of haploid mycelia into two or more morphologically indistinguishable classes which are cross-fertile but self-sterile. In a few species of fungi such as in *Hypomyces solani f. cucurbitae* (Hansen and Snyder, 1946), this incompatibility system has been found in conjunction with sexual differentiation but these occurrences are believed to be rare in the fungi.

Mating systems based solely on incompatibility are the most common in the fungi, being found in the majority of the Ascomycetes and almost all of the Basidiomycetes. These systems vary considerably in design, the most elaborate probably being that of tetrapolar sexuality as it is known to exist in the majority of the Basidiomycetes. In these fungi mating type is determined by two unlinked loci 'A' and 'B' which are comprised of two and three "subunits" respectively, each of which possesses a series of alleles.

Since mating is prevented between common-A and/or common-B individuals, inbreeding is restricted and therefore outbreeding is enhanced. Other Basidiomycetes possess a bipolar mechanism: a multiple allelic series at a single locus.

Finally, there is the relatively more simple one-locus, two-allele system found in most of the filamentous Ascomycetes including *Neurospora crassa*. This genus, belonging to the Order Sphaeriales, was named and first described by Shear and Dodge (1927). Dodge (1930, 1931a) subsequently demonstrated the species to be heterothallic with a pair of alleles segregating at meiosis to produce, in the 8-spored ascus, 4 spores of one mating type* and 4 of the other. In addition, in this organism a second genetic control (independent from the incompatibility system) operates to some extent in that female organs (ascogonia) are differentiated whereas no specific gametes are produced. Conidia, hyphae, or even trichogynes are able to fertilize the ascogonium providing they are of the opposite mating type (Dodge, 1928, 1932, 1935). Both mating types may behave as male and/or female but the only successful fertilizations appear to be A ♂ x a ♀ or a ♂ x A ♀.

Although little is known as to how and where the incompatibility control is imposed, it is generally agreed that the locus is of a compound nature. Whitehouse (1949) and Lewis (1954) have both suggested, on the basis of the number of processes that appear to be

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* These mating types are referred to as 'A' and 'a'. In earlier literature the symbols 'A' and 'B' can be found for the designation of mating types (Dodge, *loc. cit.*) as well as (+) and (-) signs (Lindegren, 1933).

controlled (directly or indirectly) by the mating type factors, that these factors are likely to constitute more than single genes. Similarly, Burnett (1956) suggests the possibility that the mating type factors are"supergenes"which act as 'switch' genes in triggering the action of a number of other genes controlling the total mating reaction. There is some genetic evidence for such complexity. For example, in the tetrapolar Basidiomycete, *Schizophyllum commune*, an estimate of 350 - 450 'A' alleles and 65 'B' alleles has been made (Raper, 1960).

Although the yeasts possess a relatively more simple mechanism experimental evidence suggests a compound locus. This group of organisms is heterothallic with two mating types being controlled by a pair of alleles designated 'a' and 'α' (Lindegren and Lindegren, 1943). More recent research has, however, revealed the incompatibility locus to be influenced by several factors. First of all the locus is genetically very labile, either allele mutating to the other or to a sterile type (Lindegren and Lindegren, 1944; Ahmad, 1953). Secondly, modifying genes have been found which directly affect the mating type in a variety of ways (Winge and Roberts, 1949; Hawthorne, 1963; Takihashi, 1958). Finally, Leupold (1958) has demonstrated in *Saccharomyces pombe* a low frequency (0.3%) of intragenic recombination within the mating type locus yielding self-fertile and sterile progeny. On the basis of the occurrence of intragenic recombination Leopold concluded that the mating type locus in *S. pombe* is a compound locus. It is believed that the above represents the first clearly demonstrated case of intragenic recombination within the mating type locus of a

heterothallic Ascomycete and carries considerable significance as to the possible evolution of heterothallism from homothallism (see Olive, 1958).

In the majority of the heterothallic Ascomycetes less complexity of the incompatibility locus is indicated. Although the locus is very stable and not extensively influenced by modifying factors, some variants have been described. The literature contains scattered reports of bisexuality in *Neurospora crassa*. Lindegren (1934b) reported an ascus containing four bisexual and four akaryotic spores, the mycelium from each of the bisexual spores being self-fertile. Lindegren explained this on the basis of two nuclei of opposite sex being included in each of the bisexual ascospores. St. Lawrence (see Olive, 1958) also obtained bisexuals in *N. crassa* but these were self-fertile indicating an incompatibility block to self-fertility. Weijer and Yang (1966) reported a self-sterile bisexual whose meiotic segregation pattern suggests a hybrid section of DNA with third division segregation in the ascus yielding bisexuals, 'A', and 'a' in the ratio of 1:3:4. Somatic segregation of these bisexuals yielded conidia of four mating type reactions: bisexual, 'A', 'a' and neutral. The explanation offered by these workers is an extension of the polaron hybrid DNA theory to include somatic as well as meiotic recombination. A compound structure of the mating type locus is anticipated on the basis of these results. Other reports of bisexuality are explained on the basis of chromosomal aberrations (Newmeyer, 1965) and disomy (Martin, 1959).

Similar evidence can be found in other heterothallic Ascomycetes. In *Cochliobolus heterostrophus*, Nelson (1957) found a single-ascospore culture which produced sterile asci when selfed and another which was self-fertile. Olive (1958) has suggested that such a strain may result from crossing over within the incompatibility locus. In a later paper, Nelson (1959a) found that the degree of compatibility varied considerably in single spore cultures. On the basis of the results obtained he postulated a complex incompatibility locus with several modifying genes. In *Chromocrea spinulosa* self-fertility of half of the spores in the ascus has been attributed to mutation at the mating type locus (Mathieson, 1952). Finally, in *Podospora anserina* Esser (1959) has shown a second incompatibility system controlled by four loci to be superimposed over the regular incompatibility system.

Hence, it appears from the various reports cited above that the incompatibility locus in heterothallic Ascomycetes, although it may be very stable in most of the Ascomycetes, is not a simple locus. Olive (1958) proposed that the two alleles of the incompatibility locus in heterothallic Ascomycetes could be two pseudoalleles of a complex locus. In his view heterothallism could have evolved from homothallism through two separate pseudoallelic mutations. Results of El Ani and Olive (1962) add considerably to this hypothesis. They found two closely linked and phenotypically different self-sterile but cross-fertile mutants in *Sordaria fimicola* which, when crossed, revealed no recombination in 504 asci analyzed. Hence these two mutants satisfy the principles of heterothallism,

(viz. self-incompatibility but cross-compatibility) thereby lending credibility to theories which propose a compound incompatibility locus in the Ascomycetes.

Our knowledge of the means by which the incompatibility alleles function in the prevention of illegitimate matings remains extremely superficial. The first advance in this field occurred in 1932 when it was demonstrated that the function of incompatibility alleles was not related to sexual differentiation (Gwynne-Vaughan and Williamson, 1932; Drayton, 1932). Later, however, Zickler (1952) reported that trichogynes of *Bombardia lunata* were positively attracted by spermatial suspension filtrates when these were of the opposite mating type. Similarly, Esser (1959) showed that the trichogynal attraction in *Podospora anserina* was specific for unlike mating types only. Although these examples represent a clear function of the mating type alleles in terms of attraction of opposite mating types it is not likely that this function is the sole means by which illegitimate matings are blocked. In contrast it has since been shown (Bistis and Raper, 1963) that, in *Ascobolus stercorarius*, this attraction is nonspecific for mating type. Hence, this function of the incompatibility locus (i.e., to attract a structure of the opposite mating type) is not consistent in the fungi.

It is generally agreed that, in heterothallic Ascomycetes at least, union of incompatible male and female gametes is prevented prior to plasmogamy. Prevention of union may therefore constitute a major function of the mating type locus. Backus (1939), while

demonstrating fusion of conidia and trichogynes of opposite mating types in *N. sitophila* reported no evidence of such fusion if their sexual structures were of the same mating type. Similar results were reported by Bistis (1957) and Esser (1959) in *A. stercorarius* and *P. anserina* respectively. It is not impossible that an antibody - antigen type of reaction between the male gamete (or male functioning structure) and the female receptive organ underlies these phenomena as has been found between cells of opposite mating type of *Hansenula wingei* (Brock, 1959). Plasmogamy has, however, since been reported between like mating types of *A. stercorarius* although no karyogamy has been observed in these cases (Bistis and Raper, 1963). It therefore appears that the prevention of plasmogamy is not the primary means by which the incompatibility alleles function in preventing illegitimate matings, at least it does not constitute the only means.

At least one, and possibly three, roles of the mating type alleles has been indicated in *A. stercorarius* (Bistis, 1957; Bistis and Raper, 1963). The first such function comprises the induction of antheridia which has been clearly demonstrated. The second role, the inability of 'A' mycelium to support the normal development of fertilized 'a' apothecial tissue, is less defined and occurs early in the development of fruiting bodies. A third role involving the induction of ascogonia, is suspected but has not been conclusively demonstrated (Dodge, 1920; Bistis, 1956).

The only logical conclusion one can draw from this is that the mating type alleles exercise their control at different stages of development in different species. The possibility also remains, of course, that there is still another block common to all heterothallic fungi through which the incompatibility alleles operate. That hormonal activity is present during the early stages of the sexual reaction has been demonstrated (see Raper 1952, 1957). Esser (1966) seems to have offered a workable hypothesis with regard to the mode of interaction of incompatibility alleles. Esser proposes two models both based on incompatibility systems in higher plants. These are the complementary-stimulant and the oppositional-inhibitor models. The complementary mechanism acts through a complementary effect of the gene products of two compatible (unlike) mating types whereas the inhibitory mechanism prevents sexual interaction between individuals of the same mating type through reaction between identical gene products. Although these models lack experimental evidence they do provide a reasonable hypothesis from which to work.

From literature it is obvious that the primary genetic control is of varying complexity in different fungi and is not amenable to simple genetic analysis. Another approach to the study of sexual reproduction in fungi takes into account the secondary physiological control (Raper, 1960). This control is imposed at the level of sexual development and is responsible for controlling the sequential progression of events through plasmogamy, karyogamy and meiosis, as well as all the intermediate steps. Secondary control encompasses a rather wide array of regulating systems some of which have been

extensively demonstrated as a step by step sequence. For others only fragmentary and isolated cases of blocks in the cycle have been reported. Hence, there exists a number of genes, independent of the compatibility locus, whose products regulate the chain of sexual events. This chain starts with the bringing together of compatible sexual elements and culminates with the formation of mature sexual ascospores.

That the total sexual cycle is a progressive sequence of events is, of course, logical and was demonstrated in *Neurospora* by Dodge as early as 1935. Dodge described three distinct stages in the course of perithecial development and maturity in *N. sitophila* and *N. tetrasperma*. First is the formation of the ascogonium and the development of hyphal tissue around this to form the "incipient ascocarp". This is followed by the bringing together of nuclei of opposite mating, differentiation of wall tissue, development of ascogenous hyphae, and nuclear fusion. The final stage is a reduction division, spore delimitation, and maturity (Dodge, 1935). Backus (1939) studied in more detail the mechanics of conidial fertilization in *N. sitophila*. He found that the conidia and the trichogyne become associated about four hours after conidiation of the culture. Although it was not demonstrated, Backus suggested that there may be a chemotropic attraction of the trichogyne for the conidium. Subsequent to association, the conidium and trichogyne fuse and at about 12 hours the majority of the conidial protoplasm has entered the trichogyne through a narrow cytoplasmic bridge. Nuclei could not be distinguished in the preparation but development of the

perithecium at 12 - 15 hours was taken as evidence that nuclear migration through the trichogyne to the ascogonium had taken place. Fusion of trichogynes and conidia of the same mating type was not observed.

A more detailed account of the sequential nature of the sexual cycle has been reported in the dioecious water mold, *Achlya*, by Raper (1951, 1957). When grown separately male and female strains do not differentiate sex organs. However, when grown together the male produces antheridial hyphae, induced by a hormone complex, the so-called A complex. In addition these hyphae secrete another hormone, B, which induces the production of oogonial initials on the female. The oogonial initials, in turn, secrete hormone C which directs the growth of antheridial hyphae towards the oogonial initials and subsequently delimits the antheridial hyphae. Hormone D, secreted by the male antheridia, now causes delimitation of the oogonia and the production of female gametes, the oospheres. This is followed in succession by the growth of tubes into the oogonium, the discharge of male nuclei through these tubes, fertilization, meiosis, spore delimitation and spore maturation.

Greater success in studying the sequential progression of the sexual cycle has been obtained by studying mutants which block specific developmental stages in the cycle. The most extensive analyses have been made on three Ascomycetes, *Sordaria macrospora*, *Sordaria fimicola* and *Glomerella cingulata*, all of which are homothallic and hermaphroditic. Disregarding minor differences in

the sexual cycles of these three species one can recognize, as a result of these studies, eight basic stages in the sequence (Raper, 1960): (1) ascogonial or protoperithecial formation; (2) plasmogamy; (3) dikaryosis and production of ascigerous hyphae; (4) production of asci; (5) karyogamy; (6) meiosis; (7) ascospore formation; and (8) ascospore and perithecial maturation. As would be expected, a block at a specific developmental stage prevents all development beyond that point.

The first thorough description of sterility mutants was made by Wheeler and McGahen in *Glomerella cingulata*. They described nine genes affecting sexual reproduction, the effect of these genes ranging from inability to produce perithecia to the production of normal perithecia but with aborted ascospores (Wheeler and McGahen, 1952; Wheeler, 1954). Wheeler (*loc. cit.*) showed how these mutants could be arranged as blocks in a series of steps leading to the completion of the sexual cycle. From the behavior of these mutants, Wheeler was able to define four major steps in the cycle; perithecial initiation, plasmogamy, karyogamy and meiosis. It is of some interest to note that six of the genes described blocked plasmogamy. McGahen and Wheeler (1951) demonstrated that these mutants, although self-sterile, were cross-fertile. Hence there is complementation of function in cross-matings. These studies were the first representation of a sexual process in fungi as a sequential progression of events along with demonstrated blocks in the process.

The most detailed study of sexual blocks is that by Esser and Straub (1956, 1958) in *Sordaria macrospora*. These workers described 15 genes controlling the sexual cycle in this organism and from this information they were able to define six definite stages in the cycle. Two of these genes, *c* and *r*, are responsible for differentiation of the ascogonium while an additional three, *cit*, *spd*, and *p*, control the subsequent development of the protoperithecium. The dikaryotic phase, karyogamy, and ascus formation are influenced by 3 genes, *pl*, *f*, and *l*. Three genes *s*, *min*, and *pa* are responsible for meiosis and spore formation, the *min* and *pa* genes blocking prior to meiosis whereas in the *s* strain meiosis proceeds normally. One gene, *ire*, is responsible for the linear arrangement of the ascospore maturation. Finally, two genes, *n* and *m*, control the discharge of the ascospores. Although each mutant was self-sterile (the reaction proceeding only as far as the blocked step) they were all cross-fertile with mutants in the other classes. In some cases, two or three genes were found which blocked the same step but their specific effect was different as judged by complementation tests. None of the genes was allelic.

In *Sordaria fimicola* Olive (1956) found self-sterile mutants some of which were cross-fertile. Further work by Carr and Olive (1959) described seven reproductive anomalies in this species: a partial self-sterile (*st-1*), a complete self-sterile (*st-2*), two mutants which enhance the sterility of *st-2*, one which suppresses the *st-1* gene, and two which lead to ascospore lethality at germination.

The sterility in *st-2* was found to be due to failure to anastomose while in *st-1* x *st-1* crosses anastomosis was not blocked. The conclusions drawn from their experimental work indicated that the fertility block could be due to failure to anastomose, failure of nuclear migration, lack of perithecial formation, or nuclear incompatibility.

Studies of blocks in the sexual cycle of heterothallic Ascomycetes have been less systematic and less fruitful. They consist largely of isolated reports of single blocks in the sexual cycle. As early as 1939 attention was drawn to the fact that in *N. crassa* development of protoperithecia and ascogenous hyphae was controlled by two different mechanisms because protoperithecia, but never ascogenous hyphae, were produced in the absence of the opposite mating type (Lindegren *et al.*, 1939). Other reported sexual blocks in *N. crassa* are all related to female sterility, viz., the failure to produce normal protoperithecia. Westergaard and Hirsch (1954) reported two such anomalies, lack of protoperithecia and abnormal protoperithecia, to be due to different genes. Fitzgerald (1963) showed in *N. crassa* that protoperithecial production was controlled by two genes which he referred to as *s* and *bk*. In addition Fitzgerald presents evidence that environmental factors play a significant role in female fertility.

Through studies of normal development, several stages of sexual development in *A. stercorarius* have been described (Bistis, 1956, 1957; Bistis and Raper, 1963). These include (1) induction of

antheridia; (2) induction of ascogonia; (3) directional growth of the trichogyne; (4) plasmogamy; and (5) attraction and proliferation of sheath hyphae around the ascogonium. Karyogamy and meiosis are of course additional irreducible stages. In *Cochliobolus heterostrophus*, Nelson (1959b, 1959c) reported two mutant strains which were self-sterile producing only sterile asci and sterile perithecia respectively when selfed. Delimitation of ascospores and differentiation of asci were therefore somehow blocked. A third strain produced no perithecia (Nelson, 1959d). Studies indicated that this was due to a recessive gene *i* which blocked protoperithecial production when the gene was carried by both parents. A similar gene was later reported in *C. carbonum* (Nelson, 1964). In *Ceratocystis fimbriata* it has been shown that perithecial production is dependent on the presence of two independent factors (Webster, 1967).

The physiological aspects of this control have not been precisely determined although there is evidence in some cases that it is mediated by a diffusible substance. As early as 1949 Markert had concluded that fertility between different strains of *Glomerella* was controlled by complementary factors and that these factors exerted their influence in part at least by means of a diffusible substance (Markert, 1949). Driver and Wheeler (1955) later demonstrated that a culture filtrate from a wild type stock induced perithecial production when added to a self-sterile culture. Esser and Straub (1958) found essentially the same phenomenon, viz. the occurrence of selfed perithecia of one or both parents in crosses of self-sterile but cross-fertile strains. However, no diffusible agents

could be found in the medium so they concluded the induction was intracellular. Olive (1956) described a mutant strain *C1*, which, not only was self-sterile, but also suppressed perithecial production in wild type strains. Carr and Olive (1959) showed this to be due to a diffusible substance found in the medium.

The existence of such diffusible substances in the fungi was first reported in 1924 by Burgeff who demonstrated that (+) and (-) strains of *Mucor mucedo* separated by a collodion membrane would produce zygophores in a few days and these would grow towards each other (see Raper, 1952). Since then studies of hormonal mechanisms in two species of fungi have revealed an elaborate system co-ordinating the various stages in the sexual cycle. Raper (1952, 1957) has demonstrated seven hormones in *Achlya bisexualis* and *A. ambisexualis* and Plempel (see Esser and Kuenen, 1967, pp. 89-90) has shown three sex hormones in *Mucor mucedo* to control the steps beginning with initiation of the zygophores and concluding with plasmogamy. Many other cases of hormonal influence over single stages in the sexual cycle have been reported such as in *Saccharomyces* (Levi, 1956) and in *Allomyces* from which a hormone, sirenin, has been isolated which is produced by the female gametangia and whose function is to attract the male gametes (Machlis, 1958a, 1958b).

Several sexual activities were previously discussed as functions of the mating type alleles and the possible role of hormones was mentioned in this regard. Among these, antheridial induction in *A. stercorarius* (Bistis, 1957) and directed trichogynal growth in *B. lunata* (Zickler, 1952) have both been demonstrated to be under

hormonal control. Such control, while now shown, has been suspected to play a part in ascogonial induction in *A. stercorarius* (Bistis, 1956).

In *Neurospora*, evidence for the existence of a diffusible substance has been reported a number of times. Moreau and Moruzi, in a series of papers (see Raper, 1952) presented evidence for a diffusible substance which could induce the formation of perithecia in the strain of the opposite mating type without the two compatible elements being brought together. Their evidence is based primarily on U-tube experiments, the two strains being grown in opposite arms resulting eventually in perithecia being produced in one of the arms although no growth could be found in the center of the U-tube. Dodge (1931b) repeated the experiments with negative results. Aronescu (1933, 1934) subjected the theory to genetic analysis using the U-tube technique. She analyzed about 50 asci from perithecia produced in one arm of the tube. The results demonstrated that all perithecia found resulted from the union of two compatible nuclei, one from each arm of the U-tube. Lindegren (1934a, 1936) described a bisexual, heterokaryotic, self-sterile strain in which self-fertility could be induced by the addition of a third highly fertile strain. In Lindegren's view, one of the strains used by Moreau and Moruzi was probably a self-sterile bisexual, the diffusible substance from the other strain supplying some essential substance making the bisexual a self-fertile. Finally Ito (1956) found that protoperithecia in *N. crassa* could be induced to develop into sterile fruit bodies by adding a filtrate from a culture of the opposite mating type.

From the literature it becomes clear that a thorough study of sexual stages in heterothallic Ascomycetes is still lacking. Such a study would enable a more rigid comparison of sexuality in homothallic versus heterothallic fungi. This thesis represents the first part of such a study. It describes the induction of many sterility mutants in *Neurospora crassa*, their phenotypic behavior, classification, and an attempt at determining the location of the probable genetic block in the sexual cycle. Raper (1959) states: "It is my own belief that sexuality provides one of the most critical keys, if not the most critical, to an eventual rationalization of the diversity of fungi" (p.109). In addition, it is reasonable to anticipate that the unravelling of the sexual complexities in the fungi will cast some light upon sexual processes in lower organisms in general, and upon their evolution in particular. Some value should accrue as a result of a better understanding of evolution of fungi through an understanding of the evolution of homothallism and heterothallism.

MATERIALS AND METHODS

1. *Strains*

The strains of *Neurospora crassa* used in this study were obtained from the Fungal Genetics Stock Center at Dartmouth College, New Hampshire. Description of the stocks is shown in Table I. Wild type strains were used primarily in crosses to demonstrate segregation of the mutant sterility gene and to obtain recombination data of this gene with mating type. *Pan-1* and *leu-3* strains were used for UV mutation induction. These were chosen for their high degree of fertility and non-leakiness after testing a total of 11 different biochemical mutants. *F1*$^-$ strains were used as tester stocks for mating type and sterility tests.

2. *Media Used*

(a) *Complete medium.* All cultures were maintained on standard complete medium consisting of Vogel's Medium N (Vogel, 1956) supplemented with 0.5% yeast extract, 0.5% casein hydrolysate, 0.1% standard vitamin solution and 0.005% tryptophan. Two percent agar was used to solidify the medium when necessary.

(b) *Liquid minimal medium.* Vogel's Medium N *(loc. cit.)* was used, supplemented when necessary with L-leucine or calcium pantothenate in the amount of 50 mg/1000 ml medium.

(c) *Crossing medium.* Standard crossing medium was used for all crosses. This medium is basically that of Westergaard and

Table I. Description of stock cultures used*

Stock No.	Marker	Allele number	Linkage group	Mating type	Remarks
FGSC #262	STA4 St. Lawrence Standard 74A			A	Wild type. Vegetative re-isolate by D. Newmeyer.
FGSC #533	79a St. Lawrence Standard			a	Wild type.
FGSC #45	f1	Lindegren (L)	IIR	A)	(Fluffy, little aerial (growth and non- (conidiating. Very (fertile especially (when used as the (protoperithecial parent.
FGSC #46	f1	Lindegren (L)	IIR	a)	
FGSC #1124	leu-3	R-156	IL	A)	Leucine auxotroph
FGSC #1125	leu-3	R-156	IL	a)	
FGSC #71	pan-1	5531	IVR	A)	Pantothenic acid auxotroph.
FGSC #494	pan-1	5531	IVR	a)	

* Barratt and Ogata, 1962, 1968.

Mitchell (1947) with slight modifications as outlined in the Stanford Neurospora Methods (1963). Biochemical requirements were added as necessary in the amount of 50 mg/1000 ml medium.

(d) *Sorbose medium.* This medium causes restricted colonial growth (Tatum *et al.*, 1949) and was used whenever single colony isolation or colony counts were carried out. The medium was prepared by substituting 90% of the sucrose in the medium by sorbose.

3. *Methods*

(a) *Criteria for judging sterility.* All mutants were selected on the basis of male sterility as determined by controlled crosses. Although the mutants were not completely sterile (with one possible exception) many of them were markedly reduced in fertility, producing, in some cases, only one or two mature perithecia per plate as compared to 10^3 - 10^4 for wild type crosses. In addition to reduction in the number of mature perithecia produced, criteria for judging sterility included any obvious block to complete fertility resulting in the production of immature perithecia (based on degree of pigmentation, stage of ostiole development, etc.) with or without spores. Mutants showing less drastic changes in fertility (e.g., slight but obvious reduction in fertility) were discarded. All mutants were tested several times to ensure reproducibility of results.

(b) *Mutagenesis.* Mutation induction was accomplished by UV irradiation of conidial suspensions in 100 mm x 20 mm plastic petri dishes with a 15 Watt General Electric G15T8 Germicidal Lamp. Suspensions of 5 - 7 day cultures were prepared in sterile distilled water, adjusted to a concentration of 1×10^7/ml $\pm$ 10%. Fifteen ml of this suspension was irradiated at a distance of 25 cm under constant but moderate agitation. Total time of irradiation was aimed at obtaining a survival of 0.1% or less (usually 4 - 5 minutes). The energy generated by the lamp at this distance as measured by a Westinghouse Ultraviolet Meter SM-200 was 630 ergs/mm^2/min. After irradiation desired dilutions (approximately 20 viable conidia per

plate) were plated on sorbose medium. To prevent any photoreactivation, plating was carried out under red light and plates were subsequently incubated in the dark for 12 - 24 hours. Total incubation time was 72 - 96 hours at 25° C. For those experiments conducted for the establishment of UV-survival curves, samples were drawn (during irradiation) at 1-minute intervals for the first 3 minutes followed by 30-second intervals up to a total time of 5 minutes.

(c) *Testing and selection of mutants.* Initial testing of irradiated cultures was carried out by a replica plating method (Vigfusson and Weijer, 1969). This method eliminates individual handling of the bulk of the isolates during isolation of colonies and testing for sterility. Plates of standard crossing medium were inoculated with a drop of mycelial suspension of the *fl*$^-$ strain of desired mating type and allowed to grow 4 - 5 days at 25° C by which time abundant protoperithecia had been produced. Colonies on the sorbose plates (each of which represented an irradiated conidium) were replicated directly on to the protoperithecial plates using a velvet pad (Lederberg and Lederberg, 1952). Master (sorbose) plates were then held in the freezer to prevent any further growth. After 14 - 18 days at 25° C crossing plates were inspected and any isolates showing marked reduction in fertility were picked from the master plate and grown on complete medium. These were retested by inoculating a *fl*$^-$ protoperithecial plate with 1 ml of a 5 x 10^3/ml conidial suspension of the isolates, after which time the plates were incubated for 14 days at 25° C. The resulting number and morphology of the perithecia produced were compared with a control

plate of the parental strain. To ensure that these isolates were homokaryotic, four conidial subcultures were made of each isolate and tested for sterility using conidial suspensions as outlined above. The mutant was only selected if all subcultures showed results similar to the original isolate with respect to fertility. After selection, the isolate was further tested to ascertain that it was prototrophic with the exception of the original leucine or pantothenic acid requirement.

The control crosses, carried out in order to quantitatively assess the relative behavior of the mutant strains, were conducted by inoculating protoperithecial plates with such concentration of conidia from the mutant strain so as to provide 5×10^3 viable conidia per plate. The required concentrations were determined from conidial viability tests carried out on the cultures just prior to the experiment. St. Lawrence *a* was used as the protoperithecial parent as it is less fertile (as a female strain) than the *fl*$^-$ strain making perithecial counts easier to obtain. After 14 days incubation at 25° C plates were examined and perithecial morphology and perithecial counts registered. These counts were determined by tallying and averaging all perithecia, mature and immature, in ten fields at 100X magnification.

(d) *Test crosses.* All test crosses for mating type and sterility were carried out by inoculating a small amount of dry conidia on to marked sectors of paired *fl*$^-$ *A* and *fl*$^-$ *a* protoperithecial plates similar to the method outlined by Smith (1962). These plates were scored after 14 days at 25° C.

(e) *Ascospore isolation.* Tetrads were isolated on 5% agar blocks as described by Ryan (1950). Random ascospores were similarly isolated by transferring ejected spores from the lid of the crossing plate on to the agar block. All spores were heat-shocked at 60° C for 30 minutes and allowed to germinate at 30° C. Because of reduced viability in many of these mutants, germination percentages varied considerably.

(f) *Complementation tests.* Heterokaryons were forced by growing in liquid minimal medium using equal conidial concentrations of the two appropriate parental strains, one of which carried a leucine requirement and the other a pantothenic acid requirement. To minimize the probability that resulting growth was due to inter-cellular diffusion of biochemical requirements rather than due to formation of a heterokaryon, these were transferred by means of a small conidial inoculum on to minimal medium slants. Conidia from these slants were then tested on the appropriate sorbose medium to determine relative proportions of heterokaryotic, homokaryotic *leu*$^-$, and homokaryotic *pan*$^-$ conidia (Klein, 1958). Only heterokaryons with at least 10% heterokaryotic conidia were used for complementation tests. Crosses were made by inoculating 1.0 ml of conidial suspensions of 2×10^4/ml on to prepared protoperithecial plates of the appropriate St. Lawrence strain. (Because of its non-conidiating property, the *fl*$^-$ strain cannot be employed for controlled reciprocal crosses.) After 14 days incubation at 25° C, complementation was judged by comparing the results with similar plates of the individual heterokaryon component strains as well as with the *pan*$^-$ + *leu*$^-$ heterokaryon. In most cases, results were either definitely negative or showed a

decided improvement in fertility, often equalling that of the wild type.

(g) *Recombination tests.* Crosses for recombination tests were carried out by preparing protoperithecial plates (as described above) of one of the parents and subsequently adding a conidial suspension of the other parent. These crosses were generally more successful than those made by inoculating both parents simultaneously on to opposite sides of the plate.

(h) *Trichogyne attraction tests.* Tests to determine the existence of trichogyne attraction for the conidium were carried out in a similar manner to that employed by Bistis (1956, 1957) in studies with *Ascobolus*. Blocks of agar measuring 2.0 x 2.0 x 0.5 mm were placed on plates of fl^{-} *a* strain containing abundant protoperithecia. Conidia to be tested were then transferred on to the block with an inoculating needle and the behavior of the surrounding trichogynes was observed microscopically at intervals for a period of up to 48 hours.

(i) *Microscopic examination of perithecial contents.* Microscopic examinations of perithecial contents was carried out simply by squeezing the contents of several perithecia on to a slide and viewing these under phase contrast (40X objective).

(j) *Female fertility tests.* Tests for female fertility were carried out by inoculating plates of standard crossing medium with the isolate to be tested followed by incubation at 25° C for

4 - 5 days prior to spermatization with a conidial suspension of the St. Lawrence strain of the appropriate mating type. Plates were incubated for 14 days at 25° C and inspected for production of mature perithecia. The fact that conidia do not germinate in the presence of mycelium of the opposite mating type (Backus, 1939) and therefore can only function as the spermatial strain in these crosses, was taken as sufficient assurance that the cross had taken place in the desired direction (i.e., wild type ♂ x mutant ♀). Subsequently the direction of the cross was conclusively demonstrated by using a male fertile, female sterile strain as the male parent.

RESULTS

1. *Mutagenesis*

Prior to any mutagenesis being carried out, it was deemed advisable to establish UV-survival curves for the strains employed. Irradiation was conducted as outlined under Materials and Methods. Seven replicate experiments were carried out using the *leu-3 A* strain. Subsequently one experiment was performed with each of the following strains *leu-3 a*, *pan-1 A*, and *pan-1 a*. Results from the different strains agreed well and, from the averaged survival data, a single dose-effect curve could be plotted (Fig. 1).

Mutation induction was initially planned to obtain a survival rate of approximately 5% on the expectation that such a survival rate would permit the selection of the highest yield of mutants (Hollaender and Emmons, 1941; Hollaender *et al.*, 1945). The first four experiments, using *leu-3 a* and *pan-1 a* strains, yielded survival rates ranging from 4.5% to 33.5% and, out of 4000 isolates tested, no mutations to reduced male fertility were observed. Subsequently the total UV dose was increased rather drastically by an increase in the total time of exposure. This change resulted in survival rates being reduced to 0.002% - 0.18% and yielded 3 male sterile mutants out of 4688 isolates tested (a mutation frequency of 0.064% based on the total number tested). As these mutants were not severely impaired with respect to fertility (see Table IV), the other mating type (A) was irradiated. A total of 24 samples of *leu-3 A* and *pan-1 A* were

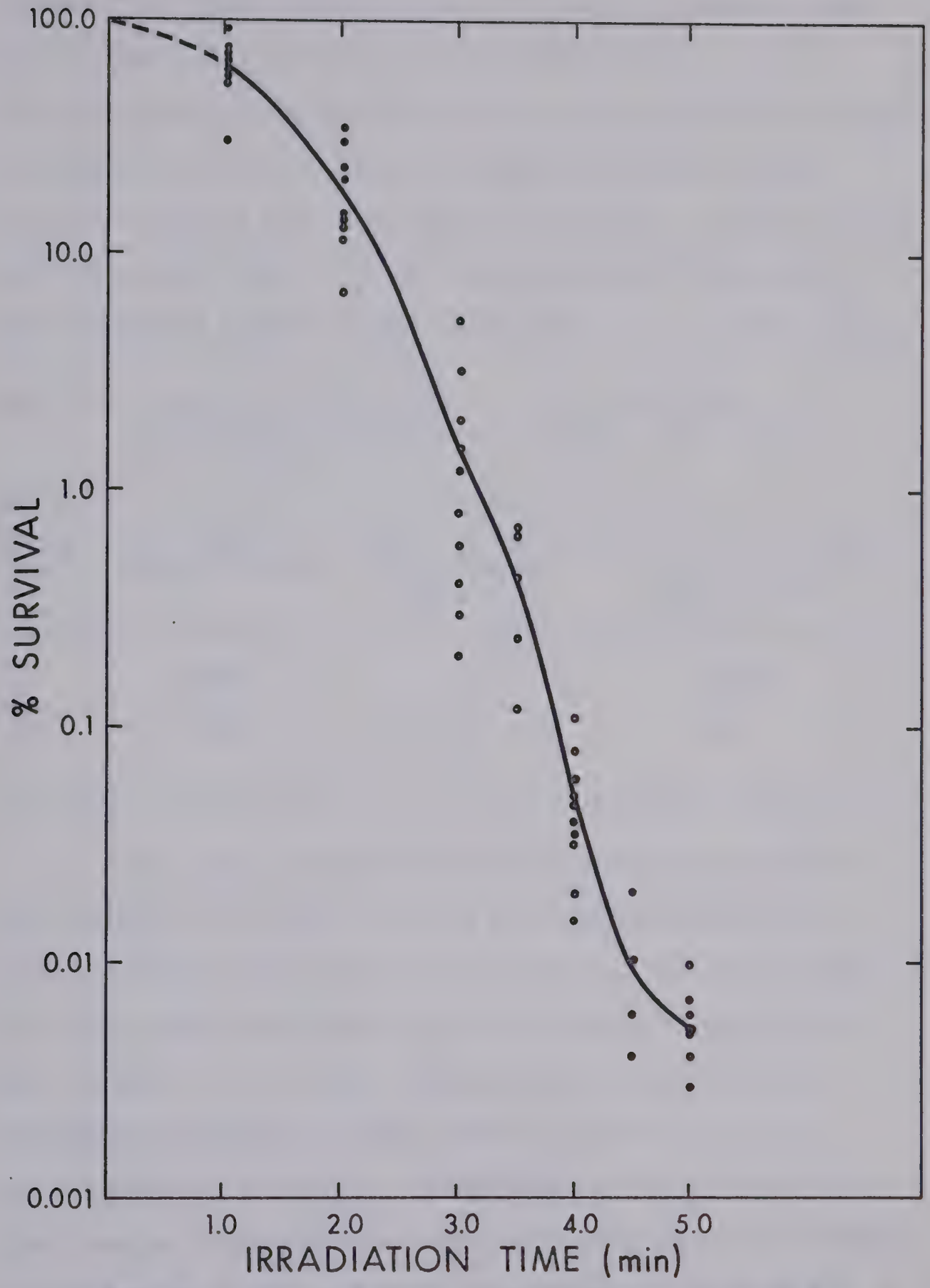


FIGURE 1 UV SURVIVAL CURVE OF Leu-3 AND Pan-1 STRAINS OF NEUROSPORA CRASSA

irradiated at slightly varying doses with resulting survival rates ranging from 0.016% to 3.4%. From the 7998 resulting isolates tested 29 mutants which displayed reduced male fertility were selected (a mutation frequency of 0.36%). A comparison of the rates of mutation to reduced male fertility (male sterility) of the two mating types is shown in Table II. The first 4 irradiated samples have been disregarded because of their comparatively high survival rates.

Table II. Comparison of the frequency of UV-induced mutation to reduced male fertility of the two mating types in *N. crassa*

Mating type	Total number of isolates tested	Number of male sterile mutants obtained	Number of male sterile mutants per 10^2 surviving cells
a	4688	3	0.064
A	7998	29	0.36

Due to the relatively low rate of mutation to sterility (particularly at the lower doses) an experiment was designed in order to obtain a correlation of total dose with mutation frequency. A 1 x 10^7 conidial suspension of *leu-3 A* was prepared and divided into 4 samples of 15 ml each. These samples were irradiated, consecutively and under the same conditions, for 3.0, 3.5, 4.0, and 4.5 minutes, respectively. Samples from each experiment were than plated on sorbose medium as previously described under conditions which prevented photoreactivation. In addition, a sample of the final experiment (4.5 min) was held in liquid minimal medium under

ordinary fluorescent light for 24 hours prior to plating to allow photoreactivation to take place. Five hundred isolates from each of the 5 lots were tested for male sterility. Results are shown in Table III and are represented graphically in Fig. 2.

Table III. Correlation of total UV dose with frequency of mutation to male sterility in *N. crassa*

Strain tested	Concentration of conidial suspension irradiated	Time of irradiation (min) 15W, 25 cm dist	% survival	Number of isolates tested	Number of male sterile mutants obtained
Leu-3 A	1 x 10^7/ml	3.0	3.4	500	1
Leu-3 A	1 x 10^7/ml	3.5	0.56	500	2
Leu-3 A	1 x 10^7/ml	4.0	0.09	500	3
Leu-3 A	1 x 10^7/ml	4.5	0.016	500	7
Leu-3 A	1 x 10^7/ml	4.5*	1.54	500	1

* With photoreactivation allowed to take place prior to plating

2. *Mutant Description*

A description of each of the mutants with respect to their crossing behavior with wild type (fl^-) as compared to the control crosses of the parental strains (from which the mutants were derived) with wild type (fl^-) is given in Table IV. These descriptions are based on the results obtained when fl^- protoperithecial plates are crossed with 1.0 ml of a 5 x 10^3 conidial suspension of the mutant as outlined under Materials and Methods. For the sake of clarity, the mutants have been classified in tentative groups according to the

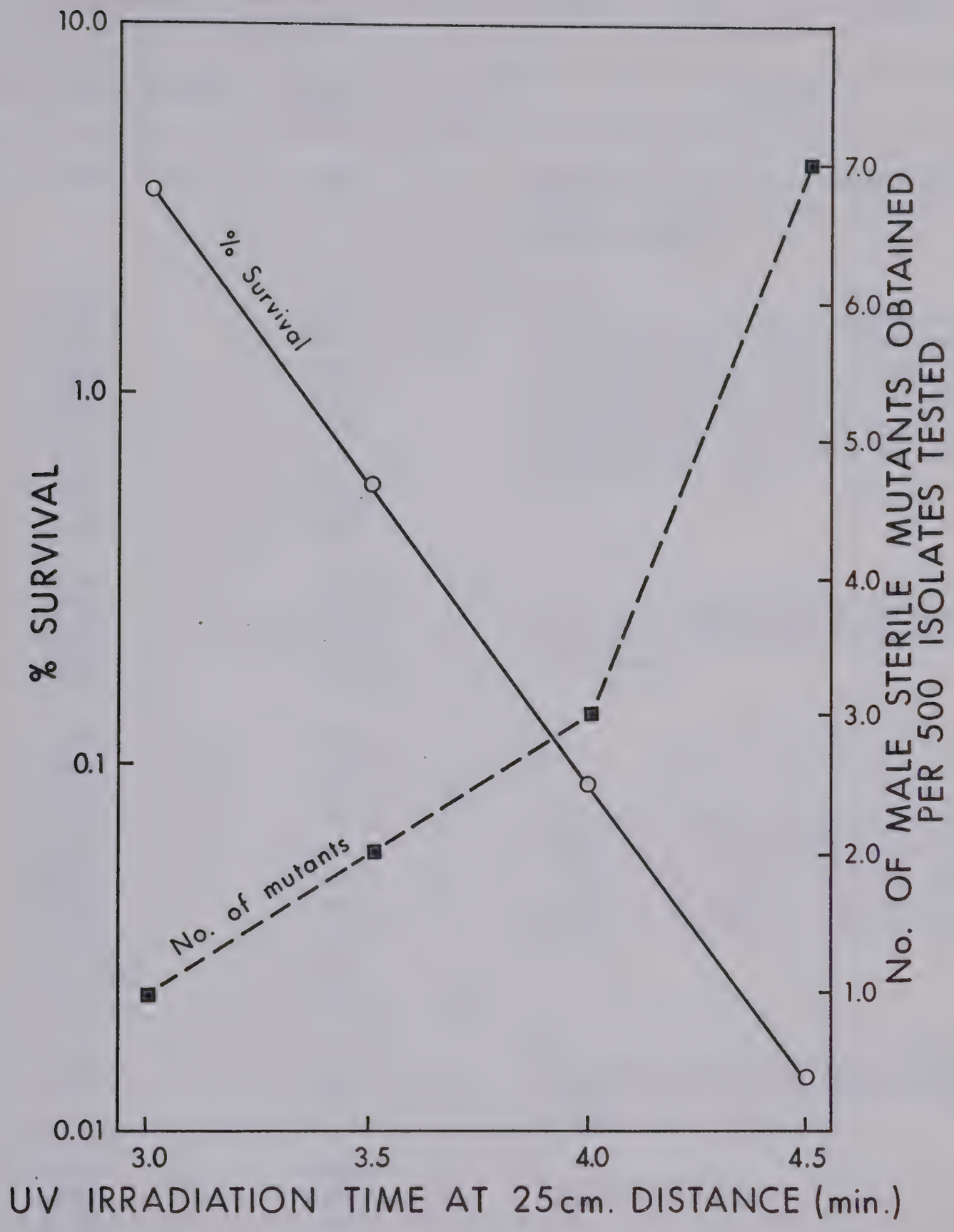


FIGURE 2. CORRELATION OF TOTAL UV. DOSE, SURVIVAL AND FREQUENCY OF MUTATION TO MALE STERILITY IN NEUROSPORA CRASSA

Table IV. Phenotypic behavior exhibited in crosses of male sterile mutants with wild type (fl^-)

Group	Isolate number	Parental strain	Mating type	Description
1	5366	Pan-1	A	Completely sterile, producing only abundant but very small, brown protoperithecia.
2	7232	Leu-3	A	)
	7384	Leu-3	A	) Abundant small, brown protoperithecia
	8455	Leu-3	A	) with occasional mature perithecia
	8553	Leu-3	A	) (0 - 10 per plate) which contain
	10299	Leu-3	A	) abundant normal spores. Strains 7384,
	10710	Leu-3	A	) 8553 and 5926 produce a slightly
	10718	Leu-3	A	) greater number of mature perithecia
	5538	Pan-1	A	) (20 - 30) per plate.
	5926	Pan-1	A	)
	16009	Pan-1	A	)
3	7065	Leu-3	A	)
	9312	Leu-3	A	) Abundant immature brown perithecia
	10233	Leu-3	A	) with no ostiole and containing
	10589	Leu-3	A	) no spores.
	10734	Leu-3	A	)
4	7341	Leu-3	A	)
	9840	Leu-3	A	)
	10402	Leu-3	A	)
	10528	Leu-3	A	) Abundant normally pigmented but
	10979	Leu-3	A	) immature perithecia as judged by
	10982	Leu-3	A	) ostiole development which is just
	11042	Leu-3	A	) starting to form. Perithecia
	16044	Pan-1	A	) empty or containing only few spores.
	P-B-13-1	Pan-1	A	)
	P-D-11-1	Pan-1	A	)
	P-D-12-1	Pan-1	A	)
5	9961	Leu-3	A	) Abundant normal perithecia but with
	10777	Leu-3	A	) spores showing a 50% abortion pattern (4-4, or 2-2-2-2).
6	12042	Leu-3	a	)
	12365	Leu-3	a	) Like Group 4 above.
	15218	Leu-3	a	)
	Leu-3 & Pan-1 parental	-	A	) 10^3 - 10^4 normal mature perithecia per plate.
	Leu-3 & Pan-1 parental	-	a	)

phenotypic pattern exhibited in crosses with wild type (fl^-). Photographs showing the appearance of the crossing plate with respect to density of perithecia of representatives from most of the groups are given in Plates 1 and 2.

3. *Segregation of Male Sterility in Crosses with Wild Type Strains*

In order to demonstrate genetic segregation of male sterility, mutants were crossed with a wild type (with respect to fertility) strain (St. Lawrence, *pan-1*, or *leu-3*). The first two, 8455 and 8553, were analyzed in more detail using random spore and unordered tetrad data. Table V shows the results obtained.

Table V. Segregation of male sterile (st^-) and mating type in random spores and unordered tetrads from crosses of st^+ *a* x st^- *A*

Isolate number	Random spores					Unordered tetrads			
	Number analyzed	Segregation of mating type and sterility				Number analyzed	Tetrad types with respect to mating type and sterility		
		a st^+	a st^-	A st^+	A st^-		PD	NPD	TT
8455	365	114	72	90	89	13	4	4	5
8553	511	206	63	57	185	12	4	0	8

It is evident from these results that, for these two mutants at least, there is little or no selection against certain genotypes in random spore isolation. Hence, random spore data provided all the essential information desired from this experiment and therefore tetrad isolation and analysis was abandoned for the rest of the mutants subjected to this experiment.

Plate I. Photographs of crossing plates showing the sexual behavior patterns of three Group 1 and 2 male sterile mutants when crossed with a wild type strain as compared to a control plate of a fertile (*Pan-1 A*) strain. All crosses were carried out by inoculating prepared protoperithecial plates with a conidial suspension (5×10^3/ml concentration) of the strain to be tested.

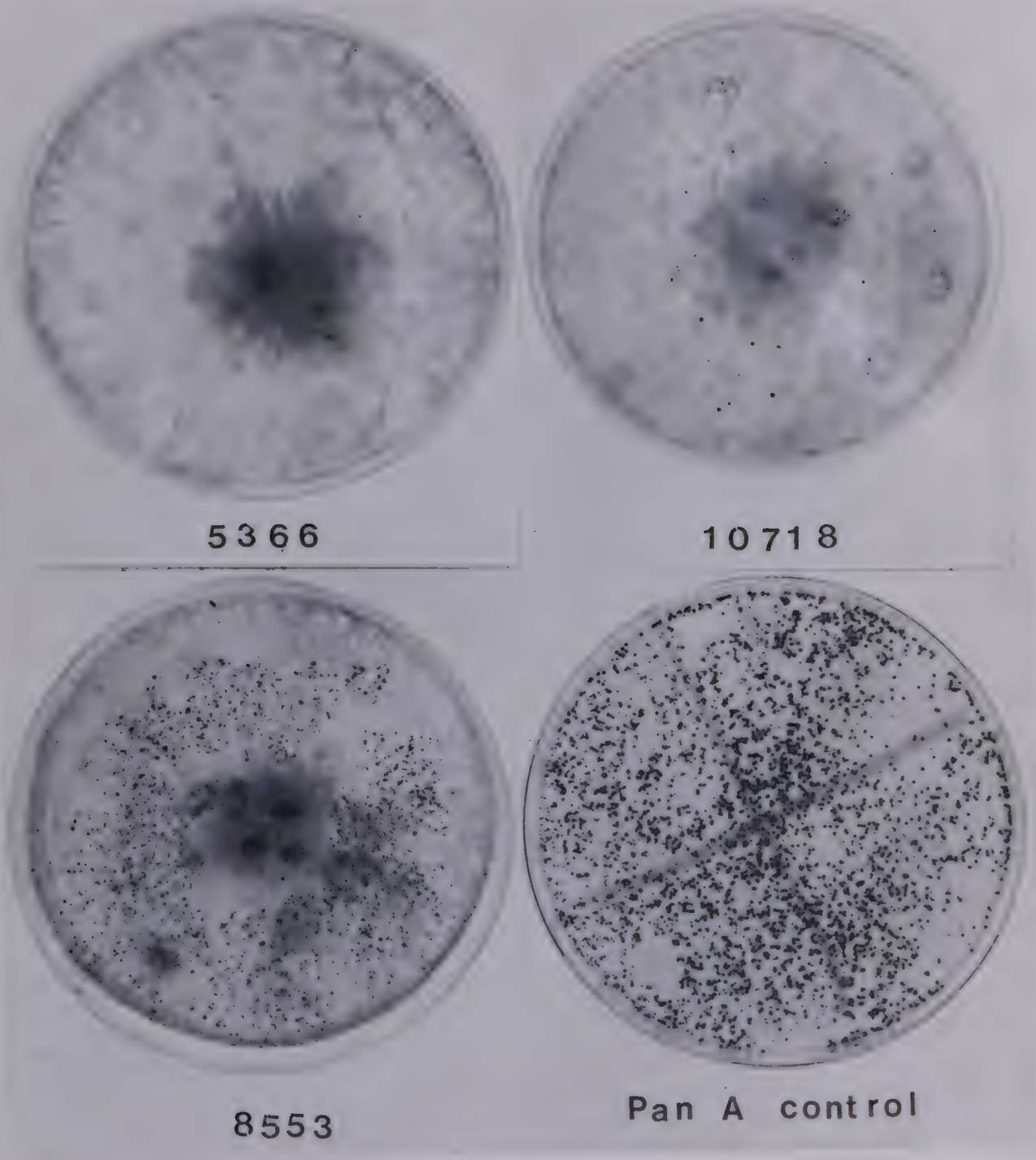
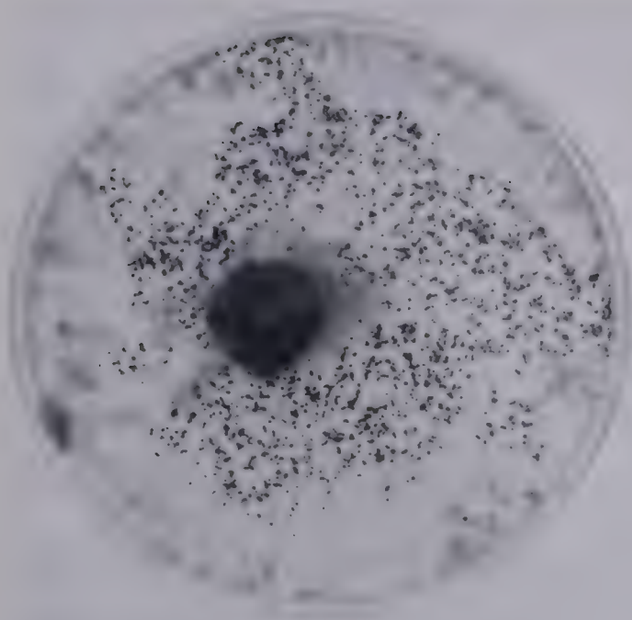
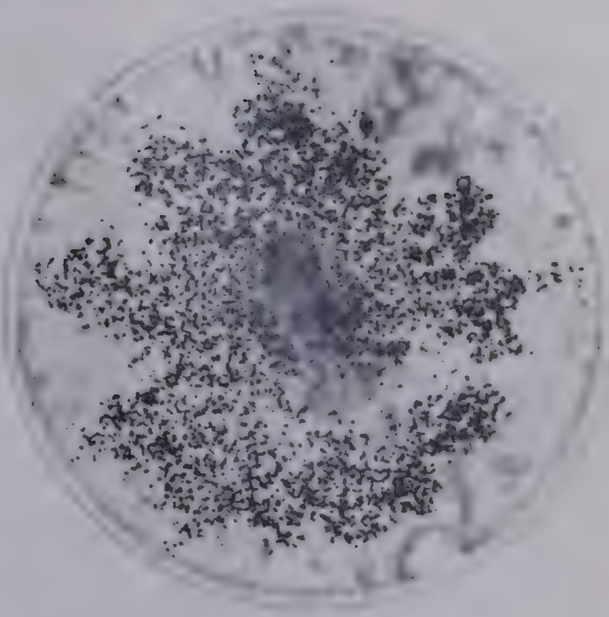

5366
10718
8553
Pan A control

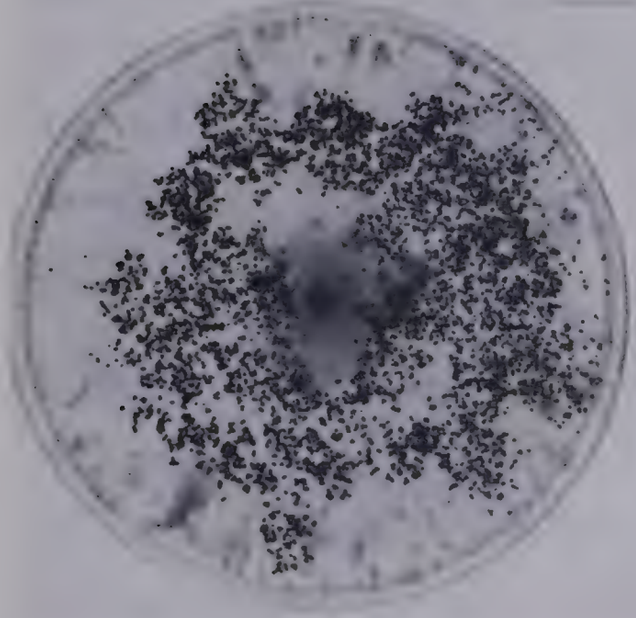
Plate II. Photographs of crossing plates showing the sexual behavior patterns of three Group 3 and 4 male sterile mutants when crossed with a wild type strain as compared to a control plate of a fertile (*Leu-3 A*) strain. All crosses were carried out by inoculating prepared protoperithecial plates with a conidial suspension (5×10^3/ml concentration) of the strain to be tested.



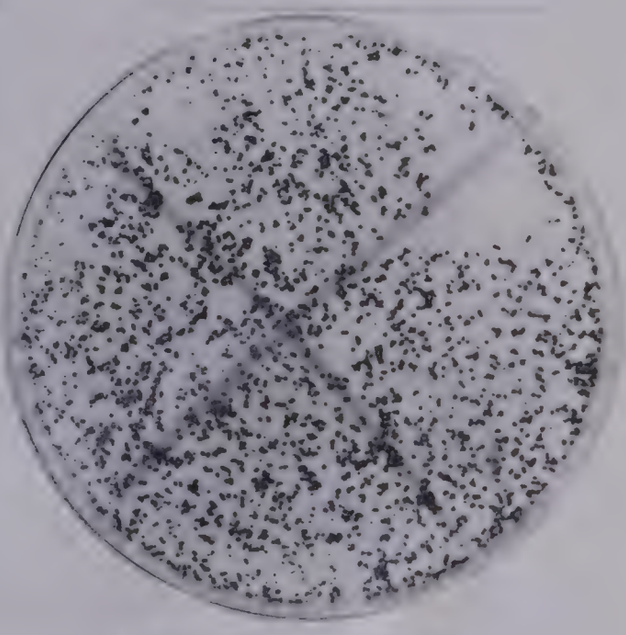
9312



9840



10402



Leu A control

Results are shown in Table VI along with ascospore germination percentages and recombination frequencies between sterility and mating type. Relative frequencies of *a vs A* and st^- *vs* st^+ are also given as an indication of the amount of selection taking place in random spore isolation.

Several of the mutants were withdrawn from further analysis at this point for various reasons. Mutants 5926, 7065, 7341, 7384 and 10734 were found to have reverted to wild type fertility. Isolate 11042 had mutated to a non-conidiator thereby making it unsuitable for further study, particularly with respect to the complementation test. Analysis of isolates 9961 and 10777 revealed that the ascospore abortion pattern also appeared in about 50% of the F_2 of normal spores indicating that the abnormality in these mutants was likely due to a chromosomal aberration (McClintock, 1945).

Although in some instances the data in Table VI reveals an equitable distribution of *a vs A* and st^- *vs* st^+, for many mutants this was, however, not the case. Some (e.g., 10982) showed a very skewed distribution pattern while others (Group 3, Group 4, and *a* mutants) yielded only male fertile progeny. To determine whether the skewed distribution was due to reduced viability of the st^- ascospores, mutant 10982 was subjected to unordered ascospore analysis. Results, shown in Table VII, strongly support an explanation on the basis of reduced viability of st^- ascospores.

Most of the mutants in Groups 3 and 4 as well as the *a* mutants, when crossed to St. Lawrence wild type, yielded only male

Table VI. Segregation of male sterility (st^-) and mating type in random spores from crosses of st^+ a x st^- A

Mutant number	Percent ascospore germination	Number of spores analyzed	Segregation of sterility and mating type				Relative frequency of		Percent recombination between male sterility (st^-) and mating type
			a st^+	a st^-	A st^+	A st^-	a : A	st^- : st^+	
5366	-	21 tetrads	15 PD:6 TT:0 NPD				-	-	-
7232	50.0	199	61	27	40	71	88 : 111	98 : 101	33.6
7384	not tested								
8455	54.6	365	114	72	90	89	186 : 179	161 : 204	45.0
8553	74.5	511	206	63	57	185	269 : 242	248 : 263	23.5
10299	55.0	220	84	36	35	65	120 : 100	101 : 119	32.2
10710	51.3	309	91	66	67	85	157 : 152	151 : 158	43.0
10718	86.0	315	103	55	54	103	158 : 157	158 : 157	31.6
5538	80.0	171	51	32	28	60	83 : 88	92 : 79	35.0
5926	not tested								
16009	75.5	169	65	28	20	56	93 : 76	84 : 85	27.8
7065	62.0	124	58	0	66	0	-	-	-
9312	73.0	146	77	0	70	0	-	-	-
10233	19.0	56	33	0	23	0	-	-	-
10589	69.0	69	30	0	39	0	-	-	-
10734	not tested								
7341	not tested								
9840	48.0	158	85	0	73	0	-	-	-
10402	49.0	162	82	0	80	0	-	-	-
10528	54.2	163	88	0	75	0	-	-	-
10979	89.0	178	86	0	92	0	-	-	-
10982	31.7	255	140	57	27	31	197 : 58	88 : 167	33.0
11042	58.0	174	87	0	87	0	-	-	-
16044	67.0	125	33	27	32	33	60 : 65	60 : 65	47.3

Table VI - continued

Mutant number	Percent ascospore germination	Number of spores analyzed	Segregation of sterility and mating type				Relative frequency of		Percent recombination between male sterility (st^-) and mating type
			a st^+	a st^-	A st^+	A st^-	a : A	st^- : st^+	
P-B-13-1	34.0	102	61	4	15	22	65 : 37	26 : 76	18.6
P-D-11-1	67.0	67	35	0	32	0	-	-	-
P-D-12-1	52.5	62	28	5	24	5	33 : 29	10 : 52	47.0
9961	72.0	90	40	0	50	0	-	-	-
10777	67.0	82	41	0	41	0	-	-	-
12042	17.5	94	18	0	76	0	-	-	-
12365	67.8	252	128	0	124	0	-	-	-
15218	51.5	181	47	0	134	0	-	-	-

Table VII. Analysis of germinated spores from 30 unordered tetrads isolated from a cross of St. Lawrence wild type x male sterile mutant 10982

Ascus	Number of spores germinated	Ascospore genotypes	Ascus	Number of spores germinated	Ascospore genotypes
1	8	2 ast^+ : 2 ast^- : 2 Ast^+ : 2 Ast^-	13	2	2 Ast^-
2	4	2 ast^+ : 2 Ast^+	14	2	2 ast^+
3	4	4 ast^+	15	2	2 ast^+
4	4	2 ast^+ : 2 Ast^-	16	2	2 ast^-
5	4	2 ast^+ : 2 Ast^-	17	2	2 ast^+
6	4	4 ast^+	18	2	2 ast^+
7	4	2 ast^+ : 2 Ast^+	19	2	2 ast^+
8	4	4 ast^+	20	2	2 ast^+
9	3	3 ast^+	21	2	2 ast^+
10	3	3 ast^+	22	1	1 Ast^+
11	3	2 ast^+ : 1 Ast^-	23	1	1 ast^+
12	3	1 ast^- : 2 Ast^+			

fertile segregants although their sterility can be maintained by vegetative subculture. These crosses, involving mutants of group 3 and 4 as well as *a* mutants, were also subjected to unordered ascospore analysis to determine whether the presence of the sterility gene was lethal to the developing ascospores. Results of this experiment are shown in Table VIII. F_1 ascospores from tetrads of 4 of these mutants were then backcrossed to wild type St. Lawrence and tetrads isolated from these crosses in order to analyze the F_2's. From each of the 8 F_1 ascospores crossed, from 1 to 3 tetrads were analyzed. Results are shown in Table IX.

Table VIII. Unordered tetrad analysis of some Group 3, 4, and *a* mutants crossed with wild type St. Lawrence

Mutant	Ascus	Number of spores germinated	Ascospore genotypes
9312	1	8	4 ast^+_+ : 4 Ast^+_+
	2	7	3 ast^+_+ : 4 Ast^+_+
	3	7	4 ast^+_+ : 3 Ast^+_+
9840	1	8	4 ast^+_+ : 4 Ast^+_+
	2	8	4 ast^+_+ : 4 Ast^+_+
	3	8	4 ast^+_+ : 4 Ast^+_+
	4	8	4 ast^+_+ : 4 Ast^+_+
	5	8	4 ast^+_+ : 4 Ast^+_+
	6	8	4 ast^+_+ : 4 Ast^+_+
	7	7	3 ast^+_+ : 4 Ast^+_+
	8	7	4 ast^+_+ : 3 Ast^+_+
	9	7	3 ast^+ : 4 Ast^+
10233	1	8	4 ast^+_+ : 4 Ast^+_+
	2	7	3 ast^+_+ : 4 Ast^+_+
	3	4	2 ast^+_+ : 2 Ast^+_+
	4	4	0 ast^+ : 4 Ast^+
10402	1	8	4 ast^+_+ : 4 Ast^+_+
	2	7	3 ast^+_+ : 4 Ast^+_+
	3	5	4 ast^+_+ : 1 Ast^+_+
	4	5	3 ast^+_+ : 2 Ast^+_+
	5	8	4 ast^+_+ : 4 Ast^+_+
	6	7	4 ast^+_+ : 3 Ast^+_+
	7	7	4 ast^+_+ : 3 Ast^+_+
	8	7	4 ast^+_+ : 3 Ast^+_+
	9	7	3 ast^+ : 4 Ast^+
10528	1	8	4 ast^+_+ : 4 Ast^+_+
	2	8	4 ast^+_+ : 4 Ast^+_+
	3	8	4 ast^+_+ : 4 Ast^+_+
	4	8	4 ast^+_+ : 4 Ast^+_+
	5	7	4 ast^+ : 3 Ast^+
10589	1	8	4 ast^+_+ : 4 Ast^+_+
	2	8	4 ast^+_+ : 4 Ast^+_+
	3	8	4 ast^+_+ : 4 Ast^+_+
	4	8	4 ast^+_+ : 4 Ast^+_+
	5	6	3 ast^+ : 3 Ast^+
10979	1	8	4 ast^+_+ : 4 Ast^+_+
	2	8	4 ast^+_+ : 4 Ast^+_+
	3	8	4 ast^+_+ : 4 Ast^+_+
	4	8	4 ast^+_+ : 4 Ast^+_+
	5	8	4 ast^+_+ : 4 Ast^+_+
	6	8	4 ast^+_+ : 4 Ast^+_+
	7	8	4 ast^+_+ : 4 Ast^+_+
	8	7	3 ast^+_+ : 4 Ast^+_+
	9	7	4 ast^+_+ : 3 Ast^+_+
	10	6	3 ast^+ : 3 Ast^+
12042	1	8	4 ast^+_+ : 4 Ast^+_+
	2	8	4 ast^+_+ : 4 Ast^+_+
	3	8	4 ast^+_+ : 4 Ast^+_+
	4	8	4 ast^+_+ : 4 Ast^+_+
	5	8	4 ast^+_+ : 4 Ast^+_+
	6	8	4 ast^+_+ : 4 Ast^+_+
	7	8	4 ast^+_+ : 4 Ast^+_+
	8	7	3 ast^+_+ : 4 Ast^+_+
	9	7	4 ast^+ : 3 Ast^+
12365	1	8	4 ast^+_+ : 4 Ast^+_+
	2	8	4 ast^+_+ : 4 Ast^+_+
	3	8	4 ast^+_+ : 4 Ast^+_+
	4	7	3 ast^+_+ : 4 Ast^+_+
	5	5	3 ast^+_+ : 2 Ast^+_+
	6	4	0 ast^+_+ : 4 Ast^+_+
	7	4	0 ast^+_+ : 4 Ast^+_+
	8	4	0 ast^+_+ : 4 Ast^+_+
	9	6	3 ast^+ : 3 Ast^+

Table IX. Unordered F_2 tetrad analysis from crosses of ascospores from F_1 tetrads x wild type St. Lawrence

Mutant	Spore No. of mutant parent	Ascus	Number of spores germinated	Ascospore genotypes
9312	1	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	2	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	3	1	8	4 ast^+_+ : 4 Ast^+_+
		2	6	4 ast^+_+ : 2 Ast^+_+
	4	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
		3	8	4 ast^+_+ : 4 Ast^+_+
	5	1	8	4 ast^+_+ : 4 Ast^+_+
	6	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	7	1	6	2 ast^+_+ : 4 Ast^+_+
	8	1	8	4 ast^+ : 4 Ast^+
10402	1	1	7	4 ast^+_+ : 3 Ast^+_+
	2	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
		3	5	3 ast^+_+ : 2 Ast^+_+
	3	1	6	4 ast^+_+ : 2 Ast^+_+
	4	1	8	4 ast^+_+ : 4 Ast^+_+
		2	6	3 ast^+_+ : 3 Ast^+_+
	5	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	6	1	8	4 ast^+_+ : 4 Ast^+_+
	7	1	8	4 ast^+_+ : 4 Ast^+_+
	8	1	7	3 ast^+ : 4 Ast^+
10528	1	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	2	1	7	3 ast^+_+ : 4 Ast^+_+
	3	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	4	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	5	1	8	3 ast^+_+ : 5 Ast^+_+
	6	1	8	4 ast^+_+ : 4 Ast^+_+
	7	1	8	4 ast^+_+ : 4 Ast^+_+
		2	6	3 ast^+_+ : 3 Ast^+_+
	8	1	8	4 ast^+ : 4 Ast^+
10589	1	1	4	2 ast^+_+ : 2 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	2	1	7	3 ast^+_+ : 4 Ast^+_+
	3	1	8	4 ast^+_+ : 4 Ast^+_+
		2	7	3 ast^+_+ : 4 Ast^+_+
	4	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	5	1	6	3 ast^+_+ : 3 Ast^+_+
	6	1	8	4 ast^+_+ : 4 Ast^+_+
		2	8	4 ast^+_+ : 4 Ast^+_+
	7	1	7	3 ast^+_+ : 4 Ast^+_+
	8	1	8	5 ast^+_+ : 3 Ast^+_+
		2	7	4 ast^+ : 3 Ast^+

Finally, reciprocal crosses were made with wild type St. Lawrence to detect any differences in the segregation of male sterility as this would indicate a cytoplasmic origin for that mutation. Although all of the Groups 3 and 4 mutants were subjected to this test, many of the crosses were found to be sterile. For the sake of comparison, 6 mutant strains which displayed a normal segregation pattern for *st*$^-$ and mating type were similarly crossed. Random spores were isolated from those crosses which yielded ascospores and results are shown in Table X.

4. *Attempts to Locate Blocks to Fertility*

In order to determine more accurately and quantitatively the relative behavior of the mutants when compared to the parental strains, controlled crosses were carried out whereby protoperithecial plates were inoculated with conidial suspensions of the mutants of such concentration to provide 5×10^3 viable conidia per plate. Details of the method have been outlined under Materials and Methods. The results of this experiment are presented in Table XI.

The possibility existed that the phenotypic behavior of some of these mutants with respect to perithecial density and development in crosses with St. Lawrence wild type was a direct result of their inability to synthesize some common nutrient requirement. An attempt was therefore made to restore fertility

Table X. Analysis of random spores from reciprocal crosses of some male sterile mutants with wild type St. Lawrence

Mutant crossed and direction of cross*	Number of spores analyzed	Segregation of mating type and sterility			
		a st$^+$	a st$^-$	A st$^+$	A st$^-$
wt. x 9312	89	49	0	40	0
9312 x wt.	57	28	0	29	0
wt. x 9840	26	15	0	11	0
9840 x wt.	132	70	0	62	0
wt. x 10402	82	40	0	42	0
10402 x wt.	80	42	0	38	0
wt. x 10982	55	28	19	3	5
10982 x wt.	42	29	5	5	3
wt. x P-B-13-1	55	30	2	10	13
P-B-13-1 x wt.	30	22	2	1	5
wt. x 8455	168	46	39	37	46
8455 x wt.	89	19	29	14	27
wt. x 8553	100	36	10	13	41
8533 x wt.	128	56	14	15	43
wt. x 10299	99	38	18	19	24
10299 x wt.	121	46	18	16	41
wt. x 10718	88	26	9	18	35
10718 x wt.	92	35	18	9	30
wt. x 5538	71	21	17	11	22
5538 x wt.	100	30	15	17	38
wt. x 16009	69	30	12	6	21
16009 x wt.	100	35	16	14	35

* The first parent listed is the protoperithecial parent.

Table XI. Density of perithecia and their morphology on plates produced by a concentration of 5×10^3 viable mutant conidia on a St. Lawrence protoperithecial strain

Mutant	Average number of perithecia per field	Description of perithecia and protoperithecia	Presence of spores	Number of mature perithecia per plate
5366	11.9	Very small, light brown	-	0
7232	20.6	Small, light brown	-*	3
8455	17.1	Small, light brown	-*	5
8553	21.4	Small, brown	-*	15
10299	20.8	Small, brown	-*	5
10710	23.7	Small, brown	-*	3
10718	21.7	Small, brown	-*	4
5538	14.8	Small, brown	-*	2
16009	6.9	Small, brown	-*	3
9312	8.4	Large, brown, no ostiole	-	0**
10528	8.9	Large, brown, no ostiole	-	0**
10233	11.0	Large, dark brown, no ostiole	-	0**
10589	14.4	Large, dark brown, no ostiole	-	0**
10979	10.0	Large, dark brown, no ostiole	-	0**
9840	9.8	Large, black, ostiole starting to form	+	0
10402	12.4	Large, black, ostiole starting to form	+	0
10982	14.1	Large, black, almost mature some with ostiole	+	0
16044	22.4	Large, black, almost mature some with ostiole	+	0
P-B-13-1	16.5	Large, black, almost mature some with ostiole	+	0
P-D-11-1	11.2	Large, black, almost mature some with ostiole	+	0
P-D-12-1	14.2	Large, black, almost mature some with ostiole	+	0
Leu^- A	26.4	Normal, mature	+	$10^3 - 10^4$
Pan^- A	21.4	Normal, mature	+	$10^3 - 10^4$

* Except in mature perithecia.

** On other occasions these mutants frequently produced a few mature perithecia in crosses with wild type.

by varying the standard crossing medium (SCM). The following variations in media were used:

1. 2.0% cornmeal plus 2.0% glucose
2. 1.5% cornmeal plus 0.2% glucose
3. SCM with glucose substituted for all of the sucrose
4. SCM with glucose substituted for ½ of the sucrose
5. SCM with glycerol substituted for all of the sucrose
6. SCM with glycerol substituted for ½ of the sucrose
7. SCM with 4.8% sodium acetate substituted for all of the sucrose
8. SCM plus 0.15% standard vitamin solution
9. SCM plus 0.005% tryptophan
10. SCM plus 0.25% yeast extract, 0.50% malt extract, 0.5% casein hydrolysate, 0.10% vitamin solution
11. SCM plus five-fold increase in the amount of leucine or calcium pantothenate added
12. SCM plus ten-fold increase in the amount of leucine or calcium pantothenate added

Using these media, crosses were made employing a wild type protoperithecial strain (St. Lawrence) and each of the mutants. In no case was there any increase in fertility of the mutants or the controls (*Pan A*, *Leu A*) over that obtained with standard crossing medium.

An experiment to test whether a variation of the amount of medium and the incubation temperature would restore fertility to any extent was devised using the same crosses described in the previous

paragraph. Crosses were carried out on 10, 15, 20, and 30 ml of standard crossing medium at incubation temperatures of 20° C, 25° C, and 30° C, for 15 days. In no case was there any significant improvement in fertility as a result of these variations. In general, the 15 ml plates incubated at 25° C produced the best results. The 10 ml and 15 ml plates dried out at 30° C and the 20° C incubation temperature appeared to be too low.

The behavior, in crosses of mutants with genetic blocks at early stages of development (mutants in Groups 1 and 2) suggested the possibility that this may be due to extremely high conidial mortality*. These mutants were therefore tested for conidial viability (percent viability being defined as the percentage of conidia which form visible colonies on sorbose medium after 4 days incubation at 30° C). Tests were carried out on conidia of the same cultures (stored at 4° C) at 4, 8, 38, and 60 days of age. Results are shown in Table XII. From the data it is evident that neither initial viability nor reduction in viability with age are seriously affected in the mutant cultures.

The relatively high proportion of mutants (initially 13 out of 32) which appear to block at early stages of sexual development indicated that either one highly mutable gene or several genes are responsible for this phenomenon. Assuming the latter possibility

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* Conidia were used as the spermatizing element in all tests for fertility.

Table XII. Conidial viability with age in male sterile strains of *N. crassa* stored at 4° C

Strain	Percent viability at the age of:			
	4 days	8 days	38 days	60 days
7232	94.0	92.0	81.0	65.0
8455	70.5	92.0	71.0	62.5
8553	78.0	80.0	72.0	70.0
10299	61.5	53.5	72.5	50.5
10710	90.0	100.0	86.5	71.0
10718	100.0	82.5	79.5	68.5
5366	88.0	89.5	82.5	73.5
5538	100.0	73.0	58.5	42.5
16009	68.5	79.5	61.5	45.0
Control (leu A)	79.0	84.5	62.0	51.0
Control (pan A)	99.5	74.0	54.5	40.0

to be the case, an attempt was made to determine more specifically the location of some of the blocks. It has been suspected (Backus, 1939) that the trichogyne is attracted chemotropically to the conidium by a diffusible substance produced by the conidium. The conidium could, therefore, through mutation, lose the ability to produce this diffusible substance. If this was the case for some of these mutants, one would expect that by increasing the concentration of spermatia (by a factor of about 10^3) partial restoration in fertility should result due to the fact that a far greater number of

conidia would be deposited upon or beside a trichogyne. A concentration of 5×10^3 conidia per plate (which was the concentration normally used in crosses) results in a density of 0.8 conidia per mm^2 of surface area of the plate whereas a concentration of 5×10^6 results in a density of 8×10^2 conidia per mm^2. Paired *fl*$^-$ protoperithecial plates were inoculated with suspensions which contained respectively, 5×10^3 and 5×10^6 conidia of the strain to be tested. All 'early' mutants were tested as well as the *leu A* and *pan A* parental strains. The criteria for judging fertility was the number of mature perithecia on the plate. Results are shown in Table XIII.

The data show an average of only a four-fold increase in the number of mature perithecia produced when the spermatial concentration is increased by a factor of 10^3 (with the highest increase for any one mutant being 6-fold) compared to an increase of 10-fold when the same test is applied to the parental strains. These results seem to indicate that none of the 'early' mutants are blocked at stages involving attraction of the trichogyne. However, to further clarify this point and to determine as to whether or not any attraction could be demonstrated in wild type strains, a test for trichogynal attraction was carried out on all the strains listed in Table XIII as well as on the St. Lawrence wild type stock. The method used has been described under Materials and Methods. In the initial test no directed growth of the trichogyne towards the conidia (or in any other direction relative to the conidia) could be observed.

Table XIII. Effect on male fertility of increasing the concentration of spermatia in crosses of wild type ♀ x mutant ♂

Strain	Number of mature perithecia produced by spermatial concentrations of:	
	5×10^3 per plate	5×10^6 per plate
5366	0	0
7232	0	3
8455	0	0
8553	12	34
10299	1	6
10710	1	4
10718	1	5
5538	2	10
16009	2	12
Average (all mutants)	2.1	8.2
Control (Leu^- A)	2,800	33,000
Control (Pan^- A)	3,200	29,000

The test was repeated using wild type *A* conidia on *fl*$^-$ *a* and *pan*$^-$ *a* protoperithecial plates as well as the reciprocal arrangement with respect to mating type. There was no indication that conidia affected the directional growth of the trichogyne. Conidia were then suspended in water prior to transferring to the block in order to avoid contamination of areas of the plate other than the agar block. Results again indicated no attraction for the conidia by the trichogyne. To eliminate any effect that light might

have had on the foregoing experiments tests were repeated in the dark. No change in the results was observed. The agar blocks were then recessed in the medium of the protoperithecial plates so that the conidia, when placed on the block, would be at approximately the same level as the mycelium. This would increase the probability that any diffusible substance emanating from the conidia would be detected by the surrounding trichogynes. Again no change in the behavior of the trichogynes was observed. Finally the test was reversed by placing conidia on to a fresh agar plate containing standard crossing medium and by inoculating intact protoperithecia of the opposite mating type approximately 0.1 mm away from the deposited conidia. Observation of the growth of the trichogynes from these protoperithecia revealed no directional growth relative to the conidia.

A very cursory attempt was then made at determining microscopically, for some of the mutants, the appearance of the asci and protoperithecia at the arrested stage of development. The method used has been described under Materials and Methods. In all of the early mutants (Groups 1 and 2) no asci or croziers could be observed. In the mutants blocking at later stages two phenotypes seemed to appear. One group displayed perithecia with young asci and croziers but no spores, while in the other group asci with 4 irregularly shaped bodies were found.

5. *Female Sterility*

All mutants were tested for female fertility to determine as to whether or not there existed any detectable correlation between female and male fertility. Tests were carried out as outlined under Materials and Methods. The results (Table XIV) revealed four different phenotypes. One of these groups exhibited complete female fertility. This group included 6 strains, all of which appear to have genetic blocks at early stages of sexual development when used as the male parent. To demonstrate that the fertility exhibited by this group of mutants was not a result of contamination in the cross, a few random spores were isolated from each of these plates and tested for male sterility. In all cases, segregation of a male sterile phenotype, similar to that of the original mutant, was demonstrated. Results are shown in Table XV.

In order to verify that female sterility does not segregate out in subsequent generations of these mutants, unordered tetrads were isolated from the testcrosses (Table XV). Four to five tetrads were analyzed in each case and no evidence for the reappearance of female sterility was observed in any of the progeny. Hence, these strains appeared to be totally female fertile.

The question then arose as to whether the degree of female fertility was correlated with the ability to produce protoperithecia in any of these mutants (Westergaard and Hirsch, 1954; Fitzgerald, 1963). All mutants were therefore tested for their ability to produce

Table XIV. Behavior of male sterile mutants with respect to female fertility in crosses with wild type

Mutant	Mating type	Male sterile group	Description
5366	A	1	)
7232	A	2	)
10710	A	2	) Female sterile, producing only
10233	A	3	) small brown protoperithecia
10528	A	4	)
8455	A	2	)
8553	A	2	)
10299	A	2	) Completely fertile, similar
10718	A	2	) to wild type
5538	A	2	)
16009	A	2	)
9312	A	3	)
9840	A	4	)
10402	A	4	) Produce abundant, pigmented
10589	A	3	) immature perithecia, some with
10979	A	4	) a few spores. Behavior similar
10982	A	4	) to mutants of male sterile
P-D-12-1	A	4	) group 4.
15218	a	6	)
16044	A	4	) Few immature perithecia
P-B-13-1	A	4	) not completely pigmented.
P-D-11-1	A	4	) Ostiole formation not
12042	a	6	) evident. No spores present.
12365	a	6	)

Table XV. Segregation of male sterility in random spores from crosses of male sterile mutant ♀ x wild type St. Lawrence ♂

Mutant strain	Number of spores analyzed	Segregation of mating type and male sterility in test crosses with wild type (fl^-)			
		a st^+	a st^-	A st^+	A st^-
8455	32	7	11	6	8
8553	36	20	1	2	13
10299	28	16	2	3	7
10718	37	17	4	5	11
5538	38	14	1	8	15
16009	37	22	3	1	11

protoperithecia (a) when grown in isolation and (b) after spermatization with wild type conidia of the opposite mating type. Unfortunately this test was not as effective as it might have been since the *leu-3* requirement, seems to cause a loss of the ability to produce protoperithecia when grown in isolation. Results are presented in Table XVI.

Tests were then conducted to determine if there was any difference in the behavior of the mutant gene with respect to female fertility when it was incorporated into the *a* genome. Two *a* male sterile recombinants from each of the mutants which block at early stages of sexual development were crossed reciprocally with St. Lawrence *A*. After incubation, plates were inspected for the degree of male fertility (to verify that they were in fact male sterile) and for

Table XVI. Female fertility of male sterile mutants determined by relative numbers of protoperithecia produced (a) in isolation after 4 days indubation at 25° C, (b) 48 hours after spermatization with wild type St. Lawrence conidia of the opposite mating type

Strain	Biochemical marker	Relative numbers of protoperithecia* produced when grown:	
		(a) in isolation	(b) after spermatization
7232	Leu-3	0	353
8455	Leu-3	0	217
8553	Leu-3	0	391
10299	Leu-3	0	379
10710	Leu-3	0	267
10718	Leu-3	0	315
5366	Pan-1	296	366
5538	Pan-1	282	285
16009	Pan-1	343	495
9312	Leu-3	0	175
9840	Leu-3	0	460
10233	Leu-3	0	410
10402	Leu-3	0	247
10528	Leu-3	0	110
10589	Leu-3	0	142
10979	Leu-3	0	340
10982	Leu-3	0	160
16044	Pan-1	346	430
P-B-13-1	Pan-1	131	251
P-D-11-1	Pan-1	178	214
P-D-12-1	Pan-1	156	256

* Obtained by counting protoperithecia in 10 fields at 100x magnification

female fertility. Results, shown in Table XVII, indicated that the re-isolates show the same sexual behavior pattern as the original mutant from which they were obtained with the exception of re-isolate 7232-10 and 7232-25 which were female fertile as compared to strain 7232 which was female sterile. Re-isolates from the other female sterile mutant, 10710, were female sterile.

The results from the female fertile mutants are, of course, as expected. Similarly the results from the 4 female sterile re-isolates (7232-10, 7232-25, 10710-95, and 10710-96) are not unusual if one assumes a double mutation (to male and female sterility) in the two original mutants (7232 and 10710) and subsequent segregation and recombination of these two genes. Assuming no linkage between the male and female sterility genes, this would result in a 1:1 segregation of female sterility among the male sterile re-isolates. In an effort to demonstrate conclusively this segregation, random spores from crosses of 7232 and 10710 with wild type St. Lawrence were isolated and analyzed for male and female fertility. Results, supporting the assumption, are shown in Table XVIII.

6. *Complementation Tests*

In an attempt to classify the sterility mutants with respect to differences in function of the genes involved, complementation tests were carried out using forced heterokaryons of the mutant strains as the male parent. Details of the method are described under Materials and Methods. Successful heterokaryons could not be obtained for all combinations of the mutant strains although the parental strains

Table XVII. Female fertility of a st⁻ recombinants from early male sterile mutants

Re-isolate number	Behavior of mutant from which re-isolate was obtained		Behavior of re-isolate	
	♂ fertility	♀ fertility	♂ fertility	♀ fertility
7232-10	-	-	-	+
7232-25	-	-	-	+
8455-9	-	+	-	+
8455-47	-	+	-	+
8553-70	-	+	-	+
8553-74	-	+	-	+
10299-2	-	+	-	+
10299-9	-	+	-	+
10710-95	-	-	-	-
10710-96	-	-	-	-
10718-8	-	+	-	+
10718-20	-	+	-	+
5538-23	-	+	-	+
5538-97	-	+	-	+
16009-13	-	+	-	+
16009-87	-	+	-	+

Table XVIII. Segregation of mating type, male sterility (st^-), and female sterility (fst^-) in random spores from cross of wild type St. Lawrence a x Ast^- fst^-

Cross	Number of spores analyzed	Segregation of mating type, st^-, and fst^-								Recombination between		
		a				A						
		st^+		st^-		st^+		st^-		m.t. and st^-	m.t. and fst^-	st^- and fst^-
		fst^+	fst^-	fst^+	fst^-	fst^+	fst^-	fst^+	fst^-			
w.t. x 7232	37	10	3	4	4	3	4	4	5	40.5%	36.5%	40.5%
w.t. x 10710	80	15	8	6	10	9	4	12	16	36.3%	48.8%	37.5%

(*leu-3* and *pan-1*) are *het*-compatible. This is believed to be attributable to a mutation of one of the *het* genes in the st^- strains. Complementation was judged on the basis of improvement in fertility of the heterokaryon over either of the components of the heterokaryon. For illustration the appearances of some of the crossing plates of the heterokaryons along with control plates of the components of the heterokaryon are shown in Plates 3 to 5. Complementation data are presented in Table XIX.

Heterokaryons of mutant strains with wild type male fertile strains were also produced and tested for male fertility to determine whether any of the mutations were dominant. Results are shown in Table XX.

Heterokaryons of all combinations of 3 female sterile strains and 3 strains which were partially female sterile were analyzed for occurrence of complementation with respect to female fertility. Results are shown in Table XXI.

7. *Recombination Tests*

The 'early' mutants were crossed in all combinations in order to obtain some information on the linkage relationships of the genes involved. St^- x st^- crosses are by and large infertile and mature perithecia were usually obtained only after several crossing attempts. In 12 of the 36 possible combinations fertile perithecia were never obtained. The recombination data are presented in Table XXII. It should be pointed out that the sample sizes upon which these data

are based are, of necessity, limited representing usually the analysis of approximately 15 tetrads or 100 random spores. Tests to determine the recombination of the female sterility genes were not conducted.

Plate III. Photographs of crossing plates of three heterokaryons of male sterile mutants which show lack of complementation, along with control plates showing the behavior of each of the components of the heterokaryon in crosses with a wild type strain. All crosses were carried out by inoculating prepared protoperithecial plates with a conidial suspension (2×10^4 concentration) of the mutant strain or the heterokaryon to be tested.

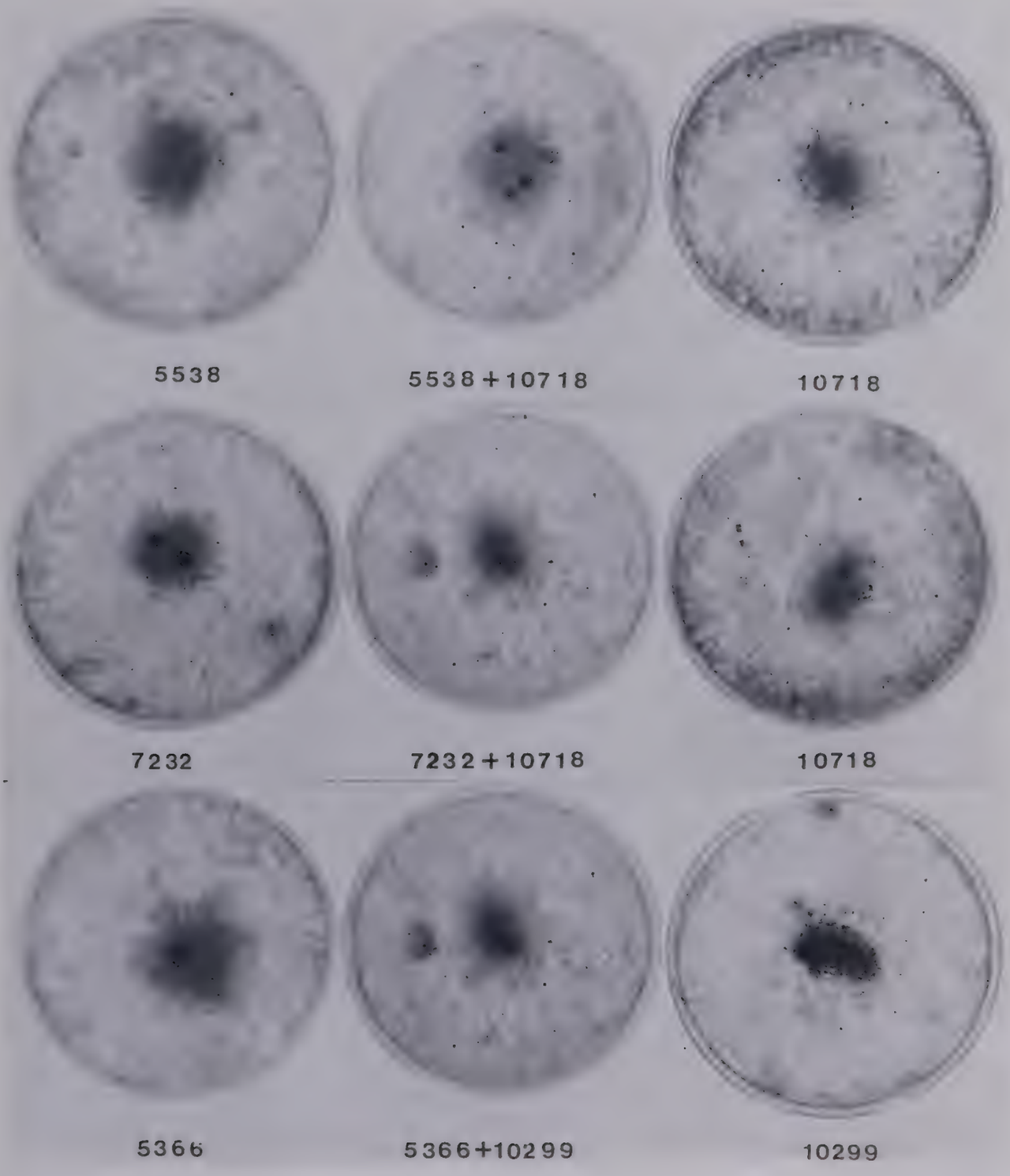

5538
5538+10718
10718
7232
7232+10718
10718
5366
5366+10299
10299

Plate IV. Photographs of crossing plates of two heterokaryons of male sterile mutants which show a high degree of complementation along with control plates of *Pan A* and *Leu A* strains in crosses with a wild type strain. All crosses were carried out by inoculating prepared protoperithecial plates with a conidial suspension (2×10^4 concentration) of the mutant strain or the heterokaryon to be tested.

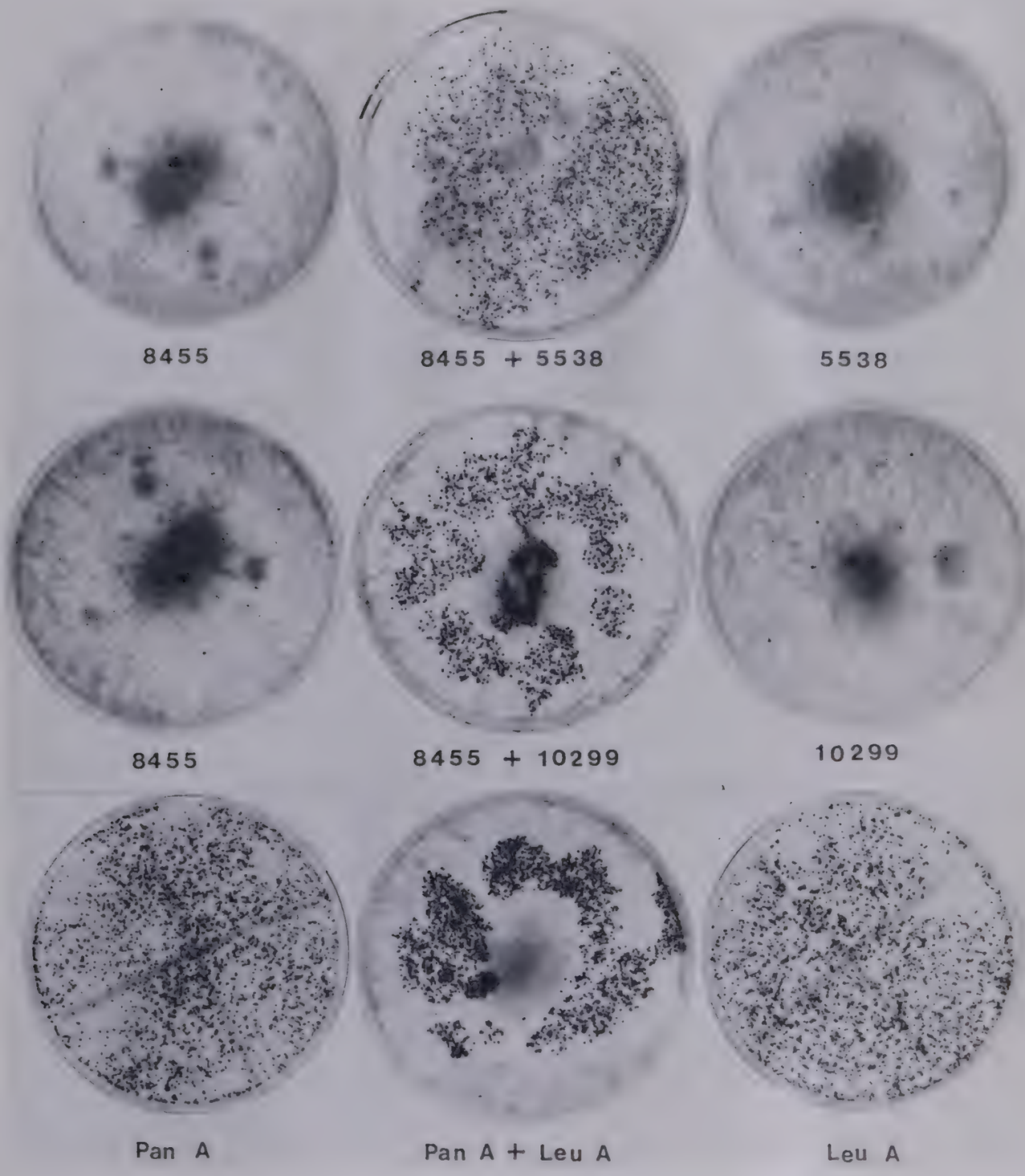
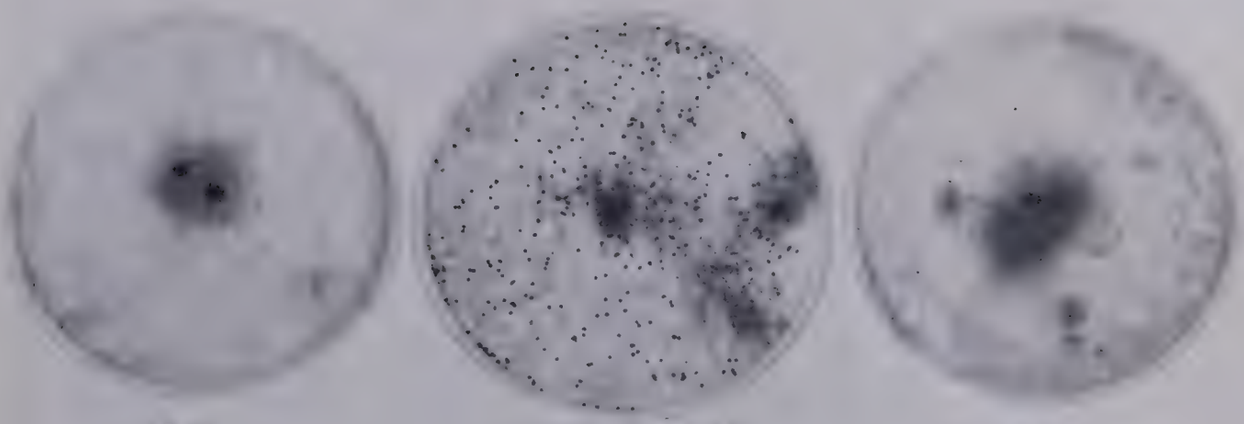
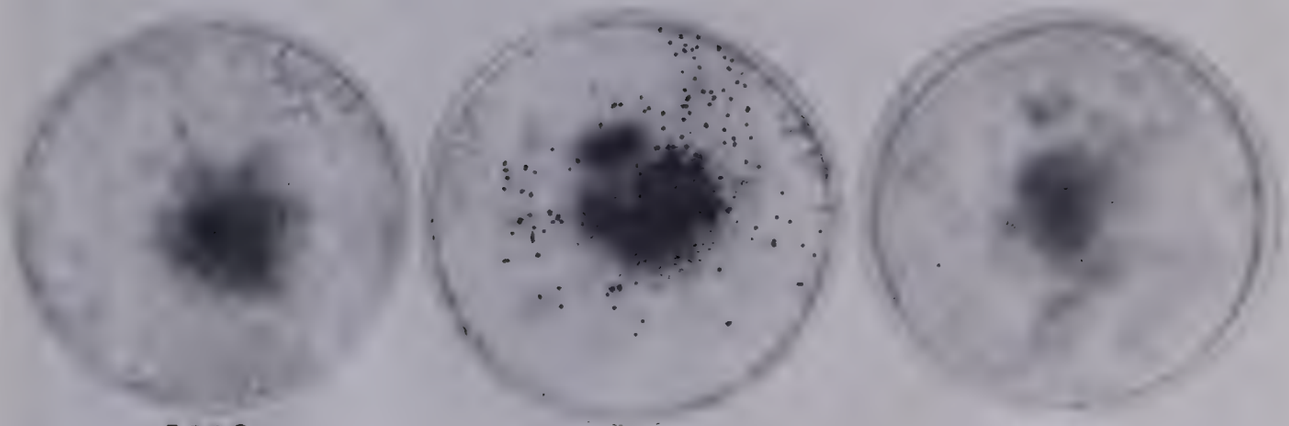

8455
8455 + 5538
5538
8455
8455 + 10299
10299
Pan A
Pan A + Leu A
Leu A

Plate V. Photographs of crossing plates of two heterokaryons of male sterile mutants which show slight but obvious complementation in crosses with a wild type strain. All crosses were carried out by inoculating prepared protoperithecial plates with a conidial suspension (2×10^4 concentration) of the mutant strain or the heterokaryon to be tested.



7232 7232 + 8455 8455



5366 5366 + 10710 10710

Table XIX. Complementation data of forced (*leu-3* and *pan-1*) heterokaryons of male sterile mutants in crosses with wild type St. Lawrence strain

	8455	8553	10299	10710	10718	5366	5538	16009	16044	P-D-12-1	10979	9312	9840	10402	10528	10589	10982
7232	+	+	-	-	-	+	-	-	+	-	+	0	+	0	0	0	0
8455	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8533	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0
10299	0	-	0	-	-	+	+	+	+	+	+	0	+	0	+	+	+
10710	-	+	-	-	+	-	+	+	+	+	0	0	0	0	0	0	0
10718	0	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
5366	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
5538	-	+	+	+	+	+	+	+	+	+	0	0	+	0	+	0	0
16009	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0
16044	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P-D-12-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

0 = Successful heterokaryons not obtained.

+ = Occurrence of complementation

Table XX. Male fertility of forced (*leu-3* and *pan-1*) heterokaryons of male sterile and fertile strains

Components of heterokaryons	Male sterile strains												
	7232	8455	8553	10299	10710	10718	5366	5538	16009	16044	P-D-12-1	9840	10982
Pan⁻ A	0	+	+	+	0	+	0	+	+	+	+	+	+
Leu⁻ A	+	+	+	+	0	+	+	+	+	+	+	0	0

0 = Successful heterokaryons not obtained.

+ = Occurrence of complementation

Table XXI. Complementation data of forced female sterile (*leu-3* and *pan-1*) heterokaryons when used as female parent in testcrosses with wild type St. Lawrence

	10710	5366	16044	P-D-12-1	10979
7232	-	-	-	-	-
10710	+	+	0		0
5366	-		0		+
16044	-				-
P-D-12-1	-				

0 = Successful heterokaryons not obtained.

+ = Occurrence of complementation

Table XXII. Occurrence of recombination in different st^- x st^- crosses

	8455	8553	10299	10710	10718	5366	5538	16009
7232	+	+	0	0	0	+	-	-
8455	+	+	+	+	0	+	+	
8553	+	+	+	+	+	+	+	
10299	0	-	0	-	0			
10710	0	+	-	0				
10718	0	-	-					
5366	0	+						
5538	0							

0 = No offspring available.

+ = Recombinants isolated

DISCUSSION

Mutagenesis

The sigmoid dose-effect curve obtained for the strains employed (Fig. 1) suggests a single hit per unit inactivation for a multiunit target. This is in agreement with the generally accepted explanation of the inactivation kinetics of *Neurospora* macroconidia (Atwood and Norman, 1949). The theoretical survival curve proposed by these workers on the basis of multinucleate conidia shows an initial plateau followed by a rapid decrease in survival similar to the pattern exhibited in Figure 1. The slight levelling off at the tail of the curve (resistant tail) can likely be attributed to a small proportion of resistant nuclei among the initial surviving population.

With regard to the probable type of damage produced by the ultraviolet light, little comment can be provided as, with the possible exception of the production of pyrmidine dimers, the physical and chemical effects of ultraviolet light on nucleic acids are not thoroughly understood (Smith, 1966). However, from Table III it can be seen that exposure to visible light after irradiation increases the survival rate from 0.016% to 1.54% but markedly reduces the proportion of male sterile mutants among the survivors (1 in 500 survivors after photoreactivation compared to 7 in 500 from the same sample prior to photoreactivation). This is in disagreement with Goodgal (1950) who reported that the proportion of mutants among

the survivors was not affected by photoreactivation although the survival rate was increased. This discrepancy could be due to a difference in the type of radiation damage produced in the two cases. Setlow and Setlow (1962) have indicated that at high doses of ultraviolet irradiation 50% of the biological inactivation is due to thymine dimer formation. Kilbey and de Serres (1967), in a series of experiments involving the *ad-3* loci of *N. crassa*, found that pyrimidine dimerization can cause all the mutational types observed. It is also known (Rupert, 1960; Terry, Kilbey, and Howe, 1967; Terry and Setlow, 1967) that thymine dimers can be excised (and the DNA damage subsequently repaired) by a light dependent enzyme. Hence if the damage which led to the present mutational types was initiated by thymine dimer formation then one would expect a sharp increase in survivors as well as a decrease in the proportion of mutants among the survivors, as a result of photoreactivation. The data in Table III (comparing 4.5 min. U.V. irradiation with 4.5 min. UV irradiation plus photoreactivation) agree with this expectation. However, it should be pointed out that the above reasoning is very speculative. Furthermore, it is not known whether photoreactivating repair takes place for other forms of UV damage.

It is of interest to note that a relatively high UV dose is necessary to bring about a mutation to male sterility in *N. crassa*. Figure 2 illustrates a rapid rise in mutant yield with increase in the total dose. On the basis of the average exposure time used in all the experiments, the total output of the source amounts to 1.1×10^6 ergs per cc of suspension irradiated. In addition,

disregarding the photoreactivation experiment, only one mutant was obtained from samples in which the survival rate was in excess of 1.0%. The majority of the mutants were obtained in samples with a survival rate of 0.1% or less. By comparison, Hollaender *et al.* (1945), in a series of 37 experiments irradiating *N. crassa* microconidia at various significant wavelengths, applied doses ranging from 1.9×10^4 to 33.7×10^4 ergs/cc and obtained survival rates of 0.4% to 72.6%. Thus, the total dose employed in the present experiments with macroconidia was higher than the dose used for most UV mutagenesis experiments with microconidia as reported in the literature. This, no doubt, partially explains why there have been no reports of demonstrated mutation to sterility in *N. crassa* up to this time.

On the basis of the published literature, it is believed that the work herein reported represents an intensive search for sterility mutants in *N. crassa*. With the selection procedure employed, detection of any mutations at the mating type locus can be expected. However, from the results obtained, it appears that none of the mutations recovered exhibit changes at the mating type locus proper. This is in support of the long held theory that the 1- locus 2-allele incompatibility system as found in filamentous Ascomycetes is very stable from the point of mutability (Raper and Esser, 1964; Burnett, 1956). This, of course, does not necessarily mean that the locus is not a compound locus as suggested by Olive (1958).

From Table II it can be seen that the mutation frequency to male sterility is more than 5 times greater for the *A* mating type than it is for the *a* mating type. The fact that these mutations are probably at several different loci (other than mating type) affecting various stages of sexual development makes this observation interesting but difficult to understand. Because of the number of loci that appear to be involved, and, as far as is known, all these loci are independent of mating type, explanations based on differences in the mating type loci *per se* are felt to be unacceptable. The possibility of the st^- mutations being incompatible with the *a* genome is also unlikely on the basis of the relatively equal occurrence of the *a* st^- (male steriles) and *A* st^- genomes among recombinant ascospores.

The most favorable explanation is felt to be that the difference in mutation frequency is a result of unknown evolutionary inherent differences in the two mating types. Physiological differences between the two mating types of *N. crassa* have been reported on several occasions (e.g. Dirkx, 1949; Fox and Gray, 1950a) but these have usually been suspected of being attributable directly to the mating type alleles. However, Fox and Gray (1950b) have found genetic differences between the two mating types other than the genetic differences at the mating type locus. It is possible that additional unknown heterogeneities exist in the two mating types of *N. crassa*. Admittedly, this explanation postulates rather far-reaching differences but the data do suggest the possibility that such may be the case.

The limited studies carried out in attempting to determine where the sterility blocks were located in the different mutant strains resulted only in negative evidence (i.e., evidence as to where the blocks were *not* located). The studies on conidial viability, variation in type and amount of medium, and variation in incubation temperature were conducted to ascertain that none of the mutants were sterile as an indirect result of some other deficiency. Based on the results of these tests (and on the segregation data presented in Table VI) it can be stated with some assurance that this was not the case and hence the mutants appear to be true mutations to male sterility.

Trichogynal Attraction

The results of the trichogynal attraction experiments are interesting although in disagreement with previous reports regarding this function in other Ascomycetes. Backus (1939) suggested that the trichogynes in *Neurospora sitophila* might "seek out, as it were, the conidia" (p. 66) and assumed this likely to be due to a chemotropic attraction. Such an attraction was subsequently demonstrated in other Ascomycetes (Zickler, 1952; Esser, 1959; Bistis and Raper, 1963). It has since been suspected by most workers that this function exists in *Neurospora crassa* as well. On the basis of the studies herein reported no such attraction was demonstrable in *N. crassa*. If these results are valid then this function has been lost in the evolution of this species (or it has never evolved).

It is difficult to understand why this is so, as the contact of the conidium with the trichogyne appears therefore to be left to chance. However, it is known that the trichogyne system in *N. sitophila* is fairly extensive (Backus, 1939). Backus reported one trichogyne system which measured a total of 2000 μ in length while one single trichogyne measured 750 μ in length disregarding branches. Bistis (1956), in reference to his work and that of Backus (*loc. cit.*) stated that more branching was observed in *N. sitophila* than in *A. stercorarius*. The closely related species, *N. crassa*, is believed to possess a similar trichogyne system. This could substitute for a chemotropic attraction scheme with respect to facilitating contact of trichogyne and conidium. By comparison, in the homothallic *N. tetrasperma*, where the bringing together of two different mating types is unnecessary, the trichogyne system is believed to be absent (Colson, 1934; Dodge, 1935). Furthermore, *N. crassa* produces an abundance of spermatizing elements (conidia). In summary it is conceivable that, because of this branching system found in *N. sitophila* and probably *N. crassa*, and the abundance of spermatia produced by *N. crassa*, the function of attraction of the trichogyne for the conidium (a) has been lost if it was ever present in these species, or, (b) has failed to evolve due to lack of necessity. Since the function has been found to be present in related genera, it probably evolved somewhere in the ancestry of the Ascomycetes and the first alternative above is therefore the more likely one.

Aberrant Segregation of Male Sterile Mutants Producing Male Sterile Progeny

The mutants 10982, P-B-13-1, and P-D-12-1, when crossed to a wild type fertile strain, exhibit an abnormal pattern of segregation of st^- and mating type among the random spores (Table VI). The st^- ascospores are found far less frequently than the st^+ ascospores (a combined total for the three strains of 124 st^- : 295 st^+). This suggests that selection is taking place against the st^- ascospores. Such selection could be due to differential viability of the st^- and st^+ ascospores. The possibility also exists that it takes place at the time of random ascospore isolation if the st^+ gene resulted in a morphologically more normal ascospore and these spores were being preferentially selected for in the isolation procedure. Tetrad analysis of the cross of wild type St. Lawrence x st^- mutant 10982 (Table VII) reveals two related facts. Firstly, out of 30 asci isolated, although 23 germinated one or more spores, only one ascus germinated more than 4 spores. Secondly, among the asci which germinated 4 spores or less the relative frequency of occurrence of the st^- and st^+ genotypes is 10 st^- : 50 st^+.

Hence, the data support the explanation of this abnormal segregation pattern based on partial ascospore lethality of the st^- ascospores. The ascospore germination percentages of 31.7%, 34.0%, and 52.5% (Table VI) also uphold this view. Either the st^- gene exhibits this lethality as a pleiotropic effect, similar to

the self-sterile *l-1* and *l-2* mutants of Carr and Olive (1958) or there exists a second mutant gene which confers partial ascospore lethality. A backcross of the st^- F_1's to wild type and subsequent tetrad analysis would have differentiated between these two alternatives but unfortunately this analysis was not carried out.

A second question arises from the segregation data for these 3 mutants (Table VI). It will be noted that, for each of the three mutants, the *a* mating type is found more frequently among the germinated ascospores (a combined relative frequency of 295 *a* : 124 *A*). The tetrad data (Table VII) similarly show a frequency of 50 *a* : 18 *A* for the 10982 strain. Thus, it appears that this st^- gene is more lethal in conjunction with the *A* allele than with the *a* allele. At this point it is difficult to hypothesize as to the reason for this phenomenon.

Aberrant Segregation of Male Sterile Mutants Producing Exclusively Male Fertile Progeny

All of the Group 3 mutants and most of the Group 4 mutants (as well as the three *a* mutants) produced only male fertile progeny when crossed to a wild type fertile strain (Table VI). On the other hand, all these mutants were previously subcultured vegetatively 4 times and were found to be stable with respect to the mutant sterility phenotype. Furthermore, the unordered tetrad data (Table VIII) reveal that the absence of the mutant phenotype in the offspring is not due to an ascospore lethal gene. Disregarding contamination in

the crosses, it would appear that 3 possible explanations remain: (a) half of the F_1's in each of the asci are in fact genotypically mutants and phenotypically wild type; (b) the mutation is transmitted maternally; (c) the mutation cannot be carried through meiosis for some cytological reason.

If the first alternative is the correct one, the mutation should be recovered in the F_2 generation. Unordered tetrad analysis of the F_2 (Table IX), however, revealed that this is not so since all of the progeny of this cross are fertile and hence a complete loss of the mutation is indicated. Similarly, Table X reveals no significant difference in the segregation pattern between reciprocal crosses. This is in agreement with the data shown for the other mutants which were tested as controls. It is not likely therefore that the mutation is maternally inherited.

On the basis of the foregoing it appears that the mutation cannot be carried through meiosis although mitosis is not similarly affected. Furthermore the defect is not heritable suggesting an extrachromosomal location although a maternal inheritance pattern is not indicated. Yet, as shown in Table VIII, in crosses of these mutants with wild type, asci are found which contain 8 mature ascospores, all of which are st^+. A translocation is ruled out as a possible cause of this phenomenon as, although this cross (translocation x wild type) could result in asci with 8 viable ascospores, 4 of these spores would still carry the translocation and would therefore exhibit the same phenotype with respect to male sterility as the original mutant. An inversion may be ruled out

for the same reasoning. Furthermore, it is not felt that an inversion or translocation would exhibit the degree of sterility shown by these mutants.

On the basis of the limited data available, one might advance the possibility that the genetic block to sterility is a direct result of the inability of the mutant to complete meiosis. The mutant could, for example, be defective in some extrachromosomal structure associated with meiosis such as the spindle apparatus or centrioles. The small percentage of fertile asci, then, could be attributable to a compensating effect by the meiotic apparatus associated with the female nucleus. It is known, for example, that in some organisms at least, centriole division is not always rigid and that two or more centrioles may be found in the cell as a result of various reasons such as supernumerary divisions of the centers (Mazia, 1951, pp. 116-139 and 184-189). Thus, we have a conceivable situation where a defect in the apparatus associated with the male nucleus could be compensated for, occasionally, by the apparatus associated with the female nucleus. In such a cross some normal asci would be found with ascospores exhibiting complete male fertility. As stated, however, the reasoning is purely speculative. Furthermore, the fact that the mutants can be propagated vegetatively is unexplained unless one assumes an inherent difference in the mitotic and meiotic apparatus. Evidence that such a difference does exist has, however, been reported in the literature (Weijer *et al.*, 1963). Undoubtedly other hypotheses could be advanced which would also agree with the experimental data.

Complementation Phases with Respect to Sexual Development in a Heterothallic Ascomycete

Although complementation with respect to sexual function has been demonstrated in homothallic Ascomycetes (Wheeler and McGahen, 1952; Carr and Olive, 1959; Esser and Straub, 1958), it is believed that the underlying study represents the first demonstration of complementation, with respect to sexual development, in a heterothallic Ascomycete. In evaluating complementation in this system it is important to consider the stage of sexual cycle which appears to be blocked. Backus (1939) demonstrated the fusion of the conidium with the trichogyne and the subsequent entry of at least a portion of the conidial protoplasm. The nucleus (or nuclei) are assumed to travel down the trichogyne into the ascogonium. Since there is no reason to believe that only one of the nuclei from the macroconidium enters the trichogyne and migrates toward the ascogonium, one can suppose that all (or most) of them do so. Normally, however, only one (Sansome, 1947; Weijer and Dowding, 1960) enters the ascogonium and becomes associated with the female nucleus.

Thus, two mutant (st^-) nuclei in a heterokaryotic conidium could complement one another (with respect to loss of function) only up to a certain stage in sexual development. This point is the time at which one male nucleus (destined for fertilization) becomes dissassociated with the rest; probably this point is reached at the time of entry of the male nucleus into the ascogonium or the time of association of the male and female nuclei in the ascogonium.

From this point on in sexual development complementation between two *male* nuclei can no longer take place. However, very shortly hereafter the *male* and *female* nuclei become associated in the ascogonium. Complementation should then be possible between the male and female nuclei during all stages of sexual development beyond this point. This has been demonstrated in some homothallic Ascomycetes (e.g. Esser and Straub, 1958).

Complementation with Respect to Sexual Development for Mutants with a Genetic Block at an Early Stage of the Cycle

From the outline presented above one would expect that, for those mutants blocking the sexual cycle prior to the stage of separation of one male nucleus from the rest of the male macroconidial nuclei, complementation results should be easily comprehensible. This is the case for all of the 9 'early' mutants of Groups 1 and 2. As the data in Table XIX indicate complementation takes place in some combinations of these mutants but not in others. It is also evident that, for these mutants, no complementation can be attributed to the wild type female nucleus because under those circumstances it cannot be expected that a particular mutant (A) complements in one combination (A + B) but not in another (A + C). Furthermore, the fact that these mutants are infertile in crosses with a wild type female strain indicates that there is no complementation from the female nucleus. Hence, there is evidence that these 9 mutants are defective in some function which takes place prior to association of the male and female nuclei in the ascogonium (since there is

apparently no complementation from the female nucleus). Furthermore, this function must take place at a time when more than one male nucleus of the fertilizing macroconidium is still actively involved in the sexual process (since these mutants do complement one another in certain combinations when the heterokaryon is used as the male parent).

Complementation with Respect to Sexual Development for Mutants with a Genetic Block at a Later Stage of the Cycle

The results of the complementation experiments for the rest of the mutants tested (those which appear to block at later stages of the cycle) are somewhat less perspicuous. For example, Group 3 and Group 4 mutants behave quite differently in crosses with a wild type fertile, the Group 4 mutants apparently being blocked at later stages than the Group 3 mutants (Table IV). Yet the Group 3 mutants tested (i.e., 9312 and 10589) do not complement with the Group 4 mutants, 16044 and P-D-12-1, respectively, although all of them show complementation with most of the Groups 1 and 2 mutants wherever successful heterokaryons were obtained (Table XIX).

Other evidence indicates that the Group 3 and 4 mutants block at stages of karyogamy and later (see p. 52 and pp. 75-77). Hence, in a heterokaryon (of a Group 3 + Group 4 mutant) which functions as a *male* strain in a cross, complementation of function between the two components of the heterokaryon cannot be expected to take place. At karyogamy (and later stages), however, the male

nucleus becomes associated with the wild type female nucleus and consequently complementation from the *female* nucleus is expected to take place leading to completion of the sexual cycle. From observations, however, it is apparent that crosses between mutants of Groups 3 and 4 and a wild type female strain do not behave in the expected manner but remain infertile.

Explanations of this phenomenon based on the inability of the genes from the female nucleus to compensate for the deficient male genes are not attractive since complementation in the ascus after karogamy has been demonstrated in homothallic Ascomycetes (Wheeler and McGahen, 1952; Esser and Straub, 1958). Another possible explanation is that the genes from the female nucleus are non-functional with respect to the damaged region of the male nucleus. This suggests that there are stages of the sexual cycle in the ascus which are specifically controlled by male or female genes. There has been no report of such a phenomenon from similar studies on homothallic Ascomycetes.

A more likely explanation is felt to be that the mutant gene is dominant although this is not evident from the data contained in Table XX. The data in this table show the results of dominance tests in a heterokaryon (mutant + wild type) used as a male strain. However, the mutants herein discussed are believed to block at late stages of sexual development (i.e., stages beyond which the male nuclei are no longer associated) and therefore dominance could not be manifested in these crosses. All ascogonia into which a wild type

nucleus entered would develop normally and the cross would therefore behave much like a fertile cross. The fact that these mutants complement (see Table XIX) with the Groups 1 and 2 mutants (which provides evidence against the dominance of these mutants) can be explained. Fertile perithecia would be produced whenever a nucleus from a Group 1 or 2 mutant overcomes its block by virtue of complementation by the other component of the heterokaryon. Assuming equal distribution of the nuclei of the two components of the heterokaryon and assuming no selectivity for the 2 nuclei with respect to fertilization this could be expected in 50% of the fertilizations. Hence, half of the perithecia would be normal, mature perithecia giving the appearance of a fertile cross. In summary, it is believed, therefore, that all of the Group 3 and 4 mutants are dominant because they are not complemented by the wild type female nucleus. This, of course, is not surprising as, with the selection procedure employed, no recessive mutants which block during the later stages of the sexual cycle would be detected.

Classification of Sterility Genes

On the basis of the complementation data presented in Table XIX a revised classification of the mutants can be made. Although this data is rather scanty for many of the mutants, it does provide a clear picture for all of the 'early' (Groups 1 and 2) mutants. It appears from this data that 4 mutant genes are responsible for the early blocks in the sexual cycle. Strains 5366, 8455, and 8553 represent, respectively, 3 of these genes while strains 7232,

10299, 10710, 10718, 5538, and 16009 represent the fourth gene. The intergenic recombination data (Table XXII) are compatible with this classification. Although these recombination data are not complete and the sample size upon which the data was based was small, there are no cases where the data are in disagreement with the complementation results.

From the phenotypic behavior of, and microscopic observation of perithecia from these 'early' mutants when crossed to wild type St. Lawrence, one would suspect that the genetic block in the sexual progression was taking place somewhere prior to the association of the male and female nuclei in the ascogonium. This is based largely on the fact that perithecial development is blocked at an early stage and no croziers or asci can be seen in the perithecia (Singleton, 1953). That some of the mutants complement one another also supports the hypothesis of a block in the early stages of sexual development (see pp. 79-80).

It is not unexpected that 4 or more genes should be found controlling the stages of sexual development up to the point of association of the male and female nuclei in the ascogonium. From similar studies in related organisms and by *a priori* reasoning, at least 6 different steps in this early part of the sexual cycle in *Neurospora* can be defined. These steps involve (entirely or at least partially) functions which one would expect to be those of the male nucleus. These are: (a) production of a diffusible substance by the conidium; (b) attachment of the conidium to the trichogyne;

(c) dissolution of the cell walls of the conidium and trichogyne;
(d) entry of the conidial cytoplasm and nuclei into the trichogyne;
(e) migration of the male nucleus (nuclei) to the ascogonium; and
(f) association of the male and female nuclei in the ascogonium.
The existence of the first step is in some doubt as discussed previously (see pp. 72-73).

Esser and Straub (1958) found 8 different genes in *Sordaria macrospora* which blocked sexual development prior to ascus formation. Five of these, however, are clearly female functions and have no counterpart in this portion of the study. The other 3, *pl*, *f*, and *l*, are described by Esser and Straub as blocking somewhere during the dikaryotic phase, karyogamy and ascus formation. Wheeler (1954) reported 6 genes which block plasmogamy in *Glomerella cingulata*. Three of these, arg^1, bi^1 and th^1, behave much like the mutants discussed here as they produce "an abundance of perithecial initials, but these never develop" (Wheeler, *loc. cit.*, p. 344). Hence, the genes reported here are very similar (phenotypically) to those of Wheeler and to the *pl*, *f*, or *l* genes of Esser and Straub (*loc. cit.*). In the present study no symbols have been assigned to these genes as more meaningful designations can be made after further analysis of the mutants (by cytological and other means). At present one can only say that these 4 genes somehow block plasmogamy.

Classification of the remainder of the mutants (i.e., mutants with a genetic block at later stages of sexual development) is considerably more speculative because of the limitations of the data. On the basis of the information available (i.e., Tables IV, VI, XIX,

and cytological observations) at least 3 different genes are indicated. The Group 3 mutants (9312, 10233, and 10589) produce, in crosses with a fertile wild type (fl^-), abundant brown perithecia which are empty and show no ostiole development. Cytological observation of the contents of these perithecia reveals croziers and very young asci which are devoid of any spore-like structures. This suggests a genetic block in the sexual development just prior to or shortly after karyogamy similar to the pattern exhibited by the B^2 gene in *G. cingulata* (Wheeler, 1954) which produce perithecia containing croziers. According to Wheeler the block is believed to occur just prior to karyogamy.

The Group 4 mutants, on the other hand, produce, in crosses with a fertile wild type fl^-, abundant perithecia which are normally pigmented but still slightly immature as judged by the development of the ostiole. These perithecia are empty although some do contain a few spores. Cytological observation of the contents of these perithecia reveals asci containing four irregularly shaped bodies. The indicated genetic block in the sexual cycle is in one of the stages from meiosis up to the time of spore wall formation. These mutants behave in a manner very similar to the dw^1 gene of *G. cingulata* (Wheeler and Driver, 1953) which produces a variety of effects from morphologically abnormal ascospores to 70 - 80% ascus abortion. According to Wheeler and Driver (*loc. cit.*) the dw^1 gene blocks meiosis. Similarly Esser and Straub (1958) reported two genes in *Sordaria macrospora*, *min* and *pa*, which block meiosis and one, *s*, which blocks shortly after meiosis. It is not unlikely, however,

that the Group 4 mutants will be found to involve several genes blocking various stages from meiosis on to spore wall formation.

Considering the segregation data (Table VI) at least two different genes are suggested for this group of mutants. One of these mutant genes, besides conferring male sterility, is probably also partially lethal in the ascospore resulting in the skewed segregation pattern of st^- and mating type in crosses with a fertile wild type. Mutants 10982, P-B-13-1, and P-D-12-1 would be included in this category. The other mutant gene confers a form of male sterility which is apparently lost in the first meiosis resulting in only fertile progeny when crossed with wild type. This would include mutant strains 9840, 10402, 10528, 10979, 11042, and P-D-11-1. It is difficult to classify mutant 16044 as it behaves phenotypically like the rest of the Group 4 mutants but exhibits no irregularities with respect to the segregation of st^- and mating type in crosses with a fertile wild type strain.

The limited complementation data available for the Groups 3 and 4 mutants, however, might be interpreted to suggest that all of these strains are mutations of the same gene as no complementation was observed within each, nor between the two groups. However, as previously discussed, if these are mutations blocking development after karyogamy (and the evidence indicates that they are) then complementation in a heterokaryon which is used as one of the parental strains in a cross to wild type could not have taken place. In this instance the components of the heterokaryon have become dissociated

and only one nucleus from the heterokaryon is involved in the further sexual development. Complementation from the female (wild type) nucleus is similarly believed to be inexistent because of dominance of the mutant gene (p. 82). Consequently, it is felt that there are at least 3 genes: one gene is represented by the Group 3 mutants and the other two genes by the Group 4 mutants.

The male sterile *a* mutants cannot be classified as, aside from determining their phenotypic behavior in crosses with a fertile wild type (fl^{-}), no further studies were carried out on these strains.

Female Sterility

Finally, the female fertility tests carried out on the male sterile mutants provide some interesting information. It is important, in analyzing sexuality in a heterothallic organism such as *N. crassa*, to differentiate between those functions that appear to be carried out by the female nucleus and those that appear to be carried out by the male nucleus. Although it is believed that after karyogamy the progression of the sexual cycle may be controlled by either male or female genes or both (based on complementation tests from homothallic Ascomycetes) there are certain stages which are specific functions of only one of the mating partner. For example, the development of the ascogonium and protoperithecium are definitely female functions since they proceed in the wild type without contact from the opposite mating type. Similarly, all the stages involving the entry of the male nucleus into the ascogonium (except the trichogynal attraction

for the conidium, if this exists) would appear to be functions of the male nucleus.

Technically, a male sterile mutation is one which blocks a specific male function while a female sterile blocks a female function. On the basis of the reasoning in the previous paragraph and barring specific male and female functions after association of the two nuclei in the crozier, all male sterile and female sterile mutations in a heterothallic organism such as *N. crassa* represent blocks at early stages of development. Hence, the only true male sterile mutations obtained in this study are the mutants of Groups 1 and 2.

Esser and Straub (1958) reported two genes in *Sordaria macrospora*, *c* and *r*, which were responsible for the differentiation of the ascogonium and 3 genes, *cit*, *spd*, and *p*, which controlled the subsequent transformation of the ascogonium into a protoperithecium. Westergaard and Hirsch (1954) found 2 strains of *N. crassa* which exhibited complete lack of protoperithecia and abnormal protoperithecia respectively, while Fitzgerald (1963) presented evidence that the function in *N. crassa* is controlled by 2 genes, *s* and *bk*. However, none of the female sterile strains reported here exhibit lack of the ability to produce protoperithecia. As the data in Table XVI indicate, all of the mutants produce abundant protoperithecia either before (*pan*$^-$ strains) or immediately after (*leu*$^-$ strains) spermatization. The location of the genetic block in the female sterile mutants (Table XIV) is as yet undetermined. Possibly the mutation results

in incompatibility of the two nuclei, for example, by preventing plasmogamy as in the *a b c v* heterogenic incompatibility system in *Podospora anserina* (Esser, 1965) or by causing the disintegration of one of the nuclei in the presence of the other. This might also explain the accompanying male sterility of these mutants (if the incompatibility is reciprocal) i.e., these mutants would be equally sterile whether used as a male or female strain.

If these 'early' mutants are defective in functions which are specifically male, one would expect that they would be female fertile. This is in agreement with the data in Table XIV with the exception of strains 5366, 7232, and 10710 which are completely female sterile. Further analysis of two of these three strains (7232 and 10710) reveals that the female sterility in these strains is due to a second mutation which segregates almost independently (40.5% and 37.5%) from male sterility (Table XVIII). Mutant 5366 was not tested in this manner because of its complete female sterility and almost complete male sterility.

The complementation data with respect to the female fertility of 7232, 5366, and 10710 (Table XXI) indicate that either the female sterile mutation of strain 7232 is dominant, or, an overlapping complementation map as shown in Fig. 3 is indicated. The data in Table XVIII show similar segregation patterns for the female sterile genes of 7232 and 10710. This may be taken as indicating that the mutations are very closely linked and possibly represent mutations of different subunits of one gene. This supports the complementation

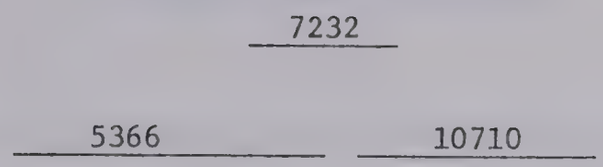


Fig. 3. Indicated complementation map for the three female sterile mutations 5366, 7232, and 10710.

map shown in Figure 3. However, further supporting evidence would be required to demonstrate this to be the case.

It is further expected that those dominant mutants which block at later stages (karyogamy *et. seq.*) of the sexual cycle, i.e., Groups 3 and 4 mutants, would exhibit female sterility patterns similar to their behavior as a male sterile strain since at the stages which are blocked, the male and female nuclei are associated. Any deviations from this would carry implications of the necessary specific functioning of either a male or female gene for certain stages of the sexual cycle. A comparison of Tables IV and XIV reveals, by and large, for the Group 3 and 4 mutants, a similar pattern of behavior in crosses whether they are employed as the male or female parent and therefore supports this view. The only exceptions to this are strains 10232 and 10589 which are completely female sterile. It is not unlikely that the complete female sterility in these strains is due to a second mutation, similar to one found in strains 7232 and 10710 (Group 2).

SUMMARY AND CONCLUSIONS

Mutations giving rise to sexual sterility were induced in *Neurospora crassa* macroconidia by ultraviolet light irradiation. Thirty-two mutants were isolated on the basis of their sterility in crosses with a wild type strain. When used as the male parent these mutants exhibited a wide spectrum of sexual behavior patterns ranging from the production of only small brown protoperithecia (complete male sterility) to the production of large and normally pigmented perithecia but with an undeveloped ostiole. These findings suggest that the sexual development cycle is blocked at various stages in the different mutant strains. All attempts to restore fertility by supplying various additives to the medium, by varying the incubation time and temperature, or by increasing the concentration of spermatia in crosses, were unsuccessful.

On the basis of complementation analysis and of limited cytological studies, 21 of these mutant strains were divided into two categories as determined by the stage of the sexual cycle in which the block occurs. These categories are: (a) 'early' mutants, i.e., mutants which block prior to the association of the male and female nuclei in the ascogonium and (b) 'late' mutants, i.e., mutants which block at karyogamy or later. Nine of the strains were 'early' mutants while twelve belonged to the category of 'late' mutants. Since no complementation appears to take place from the wild type female nucleus in crosses of the 'late' mutants with a wild type strain, dominance of these ('late) mutants is indicated.

From complementation and intergenic recombination data it can be deduced that at least four genes control the 'early' stages of the sexual cycle while a minimum of 3 genes are indicated for those mutants blocking at later stages. The 'early' mutants were, however, studied in more detail and it is likely that further analysis of the 'late' mutants will reveal additional genes controlling this phase of the sexual cycle.

Although all of the above strains were selected on the basis of their male sterility, the phenotypic behavior of the mutants when used as the female strain revealed that 5 of them were, in addition, completely female sterile. More detailed analysis of two of these male sterile, female sterile mutants demonstrated the female sterility to be due to a second mutant gene which segregated almost independently from the male sterile mutant gene. Both of these mutations block at early stages of the sexual cycle when they are used either as the spermatial or as the protoperithecial strain. By and large, the 'late' mutants, when crossed with a wild type strain, behave in much the same manner whether used as the male or the female parent in the cross. This is as expected considering the location of the genetic block in these mutant strains.

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